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Hydrogel-Electrospun Fiber Mat Composite Materials for the Neuroprosthetic Interface

Dissertation

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Philosophy in the Graduate School of The Ohio State University

By

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Abstract

Since axons do not regenerate appreciably in their native extracellular environment in the central nervous system (CNS); some researchers tried to help patients to restore their lost neural function by integrating prosthetics with their nervous system. However, achieving stable, long-term performance of implanted neural prosthetic devices has been challenging because of implantation related neuron loss and a foreign body response that results in encapsulating glial scar formation.

To improve neuron-prosthesis integration and form chronic, stable interfaces, we investigated the potential of neurotrophin-eluting hydrogel-electrospun fiber mat (EFM) composite coatings. We first synthesized and characterized diacrylate poly(ethylene glycol)-poly(ϵ -caprolactone) (PEGPCL) and poly(ethylene glycol)-poly(lactic acid) (PEGPLA) block copolymers as hydrogel materials. Then, we fabricated and evaluated poly(ϵ -caprolactone) (PCL) EFMs with different thicknesses and hydrophobicity.

Followed, we constructed PEGPCL hydrogel-PCL EFM composite materials in two different configurations using UV photo-polymerization, and compared the release kinetics of these composites using bovine serum albumin (BSA) as a model protein. The aggregation status and bioactivity of eluted proteins were also investigated. To better understand the interaction between the eluted protein and composite material, PEGPLA hydrogel-EFM composite materials were formed, comprising of EFMs with different thicknesses and hydrophobicity. The results of composite materials' swelling and release

behaviors demonstrated that both EFM's thickness and hydrophobicity had significant impact on therapeutics release profile.

In addition, we studied the cell adhesion of SK-N-SH neuroblastoma cells and rat cortical cells to hydrogel-EFM composite materials. The incorporation of external EFMs significantly enhanced cell attachment on composite materials, when compared with PEG and PEGPCL hydrogels. And RNCs preferred to adhere to composite materials composed of hydrophilic EFMs.

Also, PEGPCL hydrogel- PCL EFM composites were applied as coatings for microelectrode arrays (MEAs). Coatings were stable and persisted on electrode surfaces for over 1 month under an agarose gel tissue phantom and over 9 months in a PBS immersion bath. To demonstrate drug release, a neurotrophin, nerve growth factor (NGF), was loaded in the PEGPCL hydrogel layer, and coating cytotoxicity and sustained NGF release were evaluated using a PC12 cell culture model. Quantitative MTT assays showed that these coatings had no significant toxicity toward PC12 cells, and neurite extension at day 7 and 14 confirmed sustained release of NGF at biological significant concentrations for at least 2 weeks. Our results demonstrate that hydrogel-EFM composite materials can be applied to neuroprosthetics as a means to improve neuron-electrode proximity and enhance long-term device performance and function.

Dedication

To my husband, Shunye Gao,
and my parents, Shouli Han & Chi Yuan,
for all the love, support, and understanding you gave me.

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Publications

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Fields of Study

Major Field: Chemical and Biomolecular Engineering

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Chapter 1: Introduction

Millions of people suffer from deficits of the nervous systems due to mechanical injuries (e.g., car accidents) [1] or congenital [2] or developed neurological diseases (e.g., retinosis pigmentosa, epilepsy, stroke, Alzheimer's, Parkinson's) [1, 3]. Thus, for decades, nerve regeneration, one of the most challenging fields in science, has been extensively studied. Although the mechanism of nerve regeneration is not fully understood; the facts that nerves in the peripheral nervous system (PNS) have the potential to regenerate and the average rate of functional recovery in the PNS is $\sim 80\%$ [4] are promising. However, nerve regeneration doesn't occur in the native environment of the adult central nervous system (CNS) [5]. The physiology of the CNS presents unique challenges to neural tissue engineers in addressing nerve injuries.

Strategies for mammalian CNS nerve regeneration after CNS injury include guidance (e.g., embryonic spinal cord grafts and extracellular matrix (ECM)-based materials), biomolecular (e.g., neurotrophic factors and intrinsic neuronal factors), cellular (e.g., glial cells, macrophages, and stem cells), advanced (e.g., guidance channel fabrication techniques), and combination therapies [5]. Although some of these are promising for future work; complete functional recovery has not been observed, and the development of neural prostheses, which are designed for replacing lost neural functions rather than promoting nerve regeneration, is necessary [6, 7]. The basic idea behind neuroprosthetic devices is to create communication or interaction between the nervous

system and external devices, where neuroprosthetic interfaces [8] (e.g., brain-machine interface (BMI)) act as “bridges” between the external device and the remaining functional part of the nervous system, bypassing the damaged neurons.

One major limitation of neural prostheses’ clinical application is the biocompatibility of the neuroprosthetic interface (e.g. microelectrode arrays (MEAs)). The CNS wound healing process, which is activated by the implantation and sustained presence of MEAs, is the biggest obstacle mounted by the CNS against the implanted electrodes [9, 10]. The acute and chronic immune responses greatly compromise electrical signal transmission between implanted devices and nearby neurons, creating extraordinary difficulties in neuroprosthetic interface development [11].

1.1 Overview of the central nervous system (CNS) and CNS injury

In the body, nerve cells (neurons) and glial cells (glia) form a network comprising an organ system termed the nervous system [12]. The nervous system has two components: the central nervous system (CNS) and the peripheral nervous system (PNS) [12]. Whereas the CNS consists of the brain and spinal cord, optic, olfactory and auditory systems, the PNS is composed of ganglia and peripheral nerves lying outside the CNS [5]. Together with the PNS, which transmits signals between the CNS and the limbs and organs, the CNS is devoted to information processing by transferring the sensory input to motor output and plays a fundamental role in the control of behavior (Figure 1) [13].

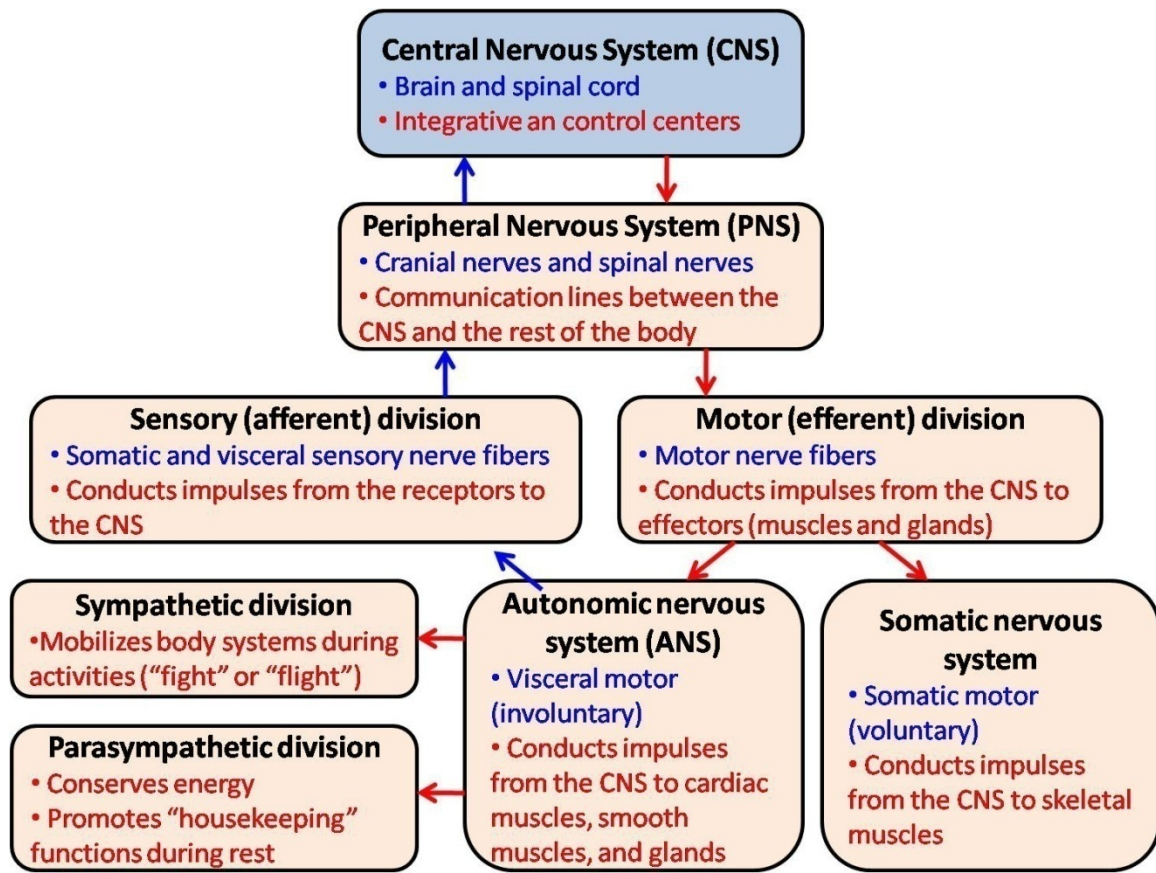


Figure 1. Simplified schema of basic structures and functions of nervous system (Reproduced from [13]).

As the critical signaling units in the nervous system, neurons can be classified functionally or structurally into different groups. However, a typical neuron is morphologically composed of four regions: the cell body, dendrites, axons, and presynaptic terminals (Figure 2) [12]. Each of these regions has a distinct role in the signaling process. The cell body, which consists of the nucleus and perikaryon, is the metabolic center making DNA, RNA, and proteins. Both dendrites and axons originate from the cell body. Whereas dendrites branch out from the cell body in a tree-like structure, receiving signal input from other neurons; the cell body also gives rise to one or more axons—the main conducting elements which can convey information (electrical

impulse) great distances (up to ~1 meter). The axon divides into fine branches, known as presynaptic terminals, which can form synaptic connections with cell bodies or dendrites of postsynaptic neurons by releasing neurotransmitter molecules to the receptive surfaces.

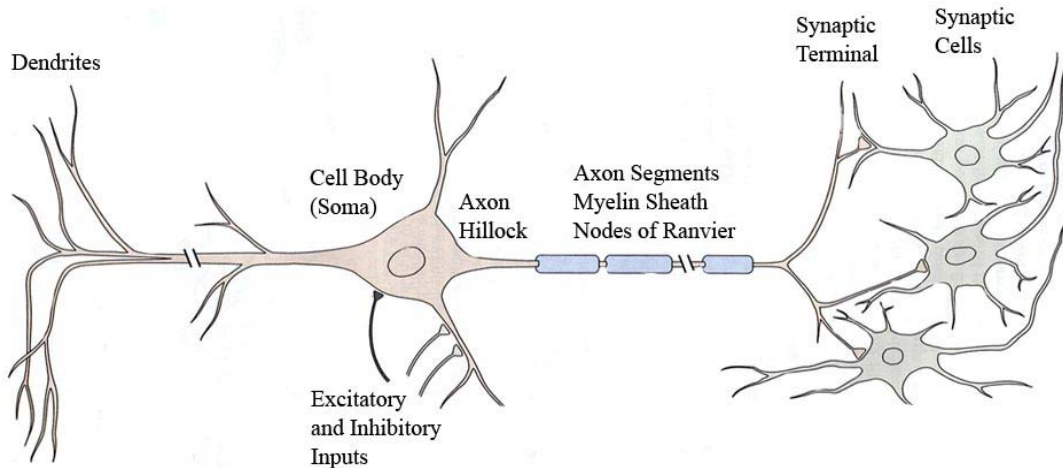


Figure 2. The structure of a typical vertebrate neuron [12].

Since neurons in the adult nervous systems are incapable of division, neuronal death caused by physical injuries usually leads to long-lasting or even permanent loss of neural functions [5, 12]. Moreover, not only do injuries that destroy the cell body of a neuron result in death, those that sever the axon of a neuron will also cause neuron degeneration and death [12]. Additionally, cells that have functional contacts (e.g., synaptic connections) with injured neurons will also degenerate [12].

In the PNS, due to the availability of neurotrophic factors such as nerve growth factor (NGF) and the presence of Schwann cells (the major component of sheaths around axons), neurons are able to regenerate their axons [12]. NGF, the best-characterized neurotrophic factor released by neurons, can promote neuron survival, growth, and axon regeneration [14, 15]. After some injuries, the recruitment of circulating macrophages

will stimulate the proliferation of Schwann cells in the vicinity, resulting in the secretion of several extracellular proteins (e.g., laminin) important for axon extension. In addition, Schwann cells may be a source of NGF to injured sympathetic and sensory neurons under certain circumstances.

In contrast to what happens in the PNS, after CNS injuries, axons can't regenerate appreciably in their native environment. This is partly due to the absence of several glycoproteins (e.g., laminin and fibronectin) in the extracellular matrix and intracellular proteins (e.g., GAP-43) that are effective in facilitating axon growth [12]. Also, some myelin-associated glycoproteins expressed in the mature CNS actively inhibit axonal regeneration [16-18]. The brain-spine barrier impedes macrophage infiltration in the CNS and delays scavenging of inhibitory myelin [19]. Additionally, the injury-mediated mechanism activates the proliferation and morphological transformation of both astrocytes and microglia, leading to the formation of an impenetrable glial scar composed of myelin, cellular debris, as well as oligodendrocytes, hypertrophic astrocytes and amoeboid microglia [11, 20] (Figure 3). The glial scar further prevents regeneration of axons. Moreover, even if nerve fibers regenerate in the CNS, it is difficult for them to traverse the PNS-CNS transition zone [21, 22].

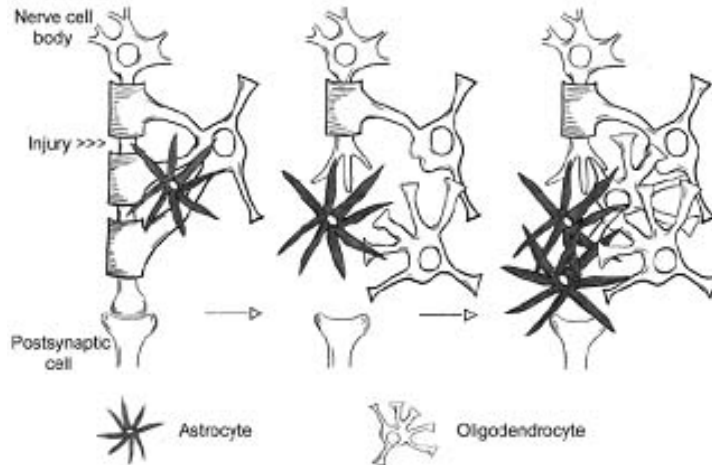


Figure 3. Responses to axotomy in the spinal cord (Reproduced from [5] and [23]).

1.2 Limitations of neuron-electronic device interfaces

Since it's challenging to completely restore lost neural function via nerve regeneration in the CNS, neuroprosthetic devices have been developed to replace damaged natural communication pathways in the nervous system [24]. For clinical applications, microscale neural probes (microelectrodes) are applied as neuron-electronic device interfaces (e.g. brain-machine interface (BMI) (Figure 4)) [25, 26]. Currently, microscale neural probes (microelectrodes) are the only approach for extracellular neural activity recording with high selectivity, which can be used in either acute/ intraoperative or chronic/ implant applications. Neural probes with multiple electrode sites (e.g., MEAs) provide the means to interface neurons at multiple, discrete positions simultaneously, and implantable neural probes allow neural recording or stimulation over multiple sessions. These electrodes can be divided into two categories: recording electrodes and stimulation electrodes [1]. Whereas the major task of the recording electrodes is to record neural activity with high signal to noise ratios (SNR) from single or multi units (individual neurons), stimulation electrodes are designed to excite nearby neurons. Current neural

probe technology aims to increase neural interface selectivity, sensitivity, fidelity (signal quality (SNR>5), stability and reliability) and bandwidth [1].

There are two primary factors that compromise chronic neural probe efficacy: 1) local tissue anatomy, which frequently prevents proximal device implantation and results in a significant separation of electrodes from target neurons [27, 28] and 2) the formation of an electrically insulating glial sheath composed of compact activated astrocytes and microglia around the device as a result of cellular and tissue responses to the sustained presence of the device [29-31].

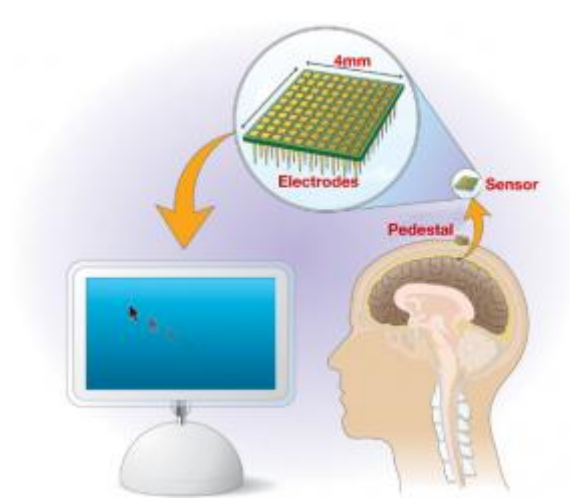


Figure 4. Demonstration of brain-machine interface (BrainGate neural interface system (<http://www.scientificamerican.com/article.cfm?id=braingate-neural-interface>)).

The distance between the electrode site and the neuron cell body plays an important role in obtaining reliable and stable electrode performance. Since spike amplitude decreases as $\sim 1/r$ (r = distance of spiking neuron to electrode) in typical situations; r should be on the order of cell dimensions (50~75 μm) [32]. Additionally, electrode performance relies greatly on the density of the healthy neurons around the electrode site.

In particular, the complex CNS immune reaction (Figure 5) to implanted probes remarkably hinders the development of the neuron-electronic device interface [10, 11, 31]. The electrode implantation process ruptures blood vessels, rips or slices the nearby neurons, activates proliferation of astrocytes, and triggers morphological change in microglia [11]. Reactive microglia are characteristic of acute inflammation, and primarily responsible for the unstable electrode performance that occurs 1-3 weeks post-implantation. Once the acute response declines, a chronic immune reaction can be observed. A more compact encapsulation sheath around the electrode is deposited by 6-8 weeks post-implantation [10, 33, 34], and remains constant for over 12 weeks [31]. The sheath, called a glial scar, is made of activated astrocytes and probably other factors and cell types. It insulates the electrode from nearby neurons, hinders biomolecule diffusion, and increases impedance [35]. Further, the glial scar produces local tissue remodeling over time, repelling neurons from the electrode sites [36, 37]. This scar is believed to be the primary cause of electrode failure and inconsistent electrode performance in a chronic setting [10, 31, 34, 37].

In addition, neuronal loss is speculated to be involved in the tissue reaction [34]. Some researchers [34, 38-40] believe that the foreign body response may also mediate neurotoxic mechanisms at the neuron-electronic device interface, leading to the formation of a “kill zone” (no neurons around the electrode site). Thus, reduction of the foreign body response and glial scar formation to obtain high electrode performance in the long term is a key challenge in the development of the neuron-electronic device interface.

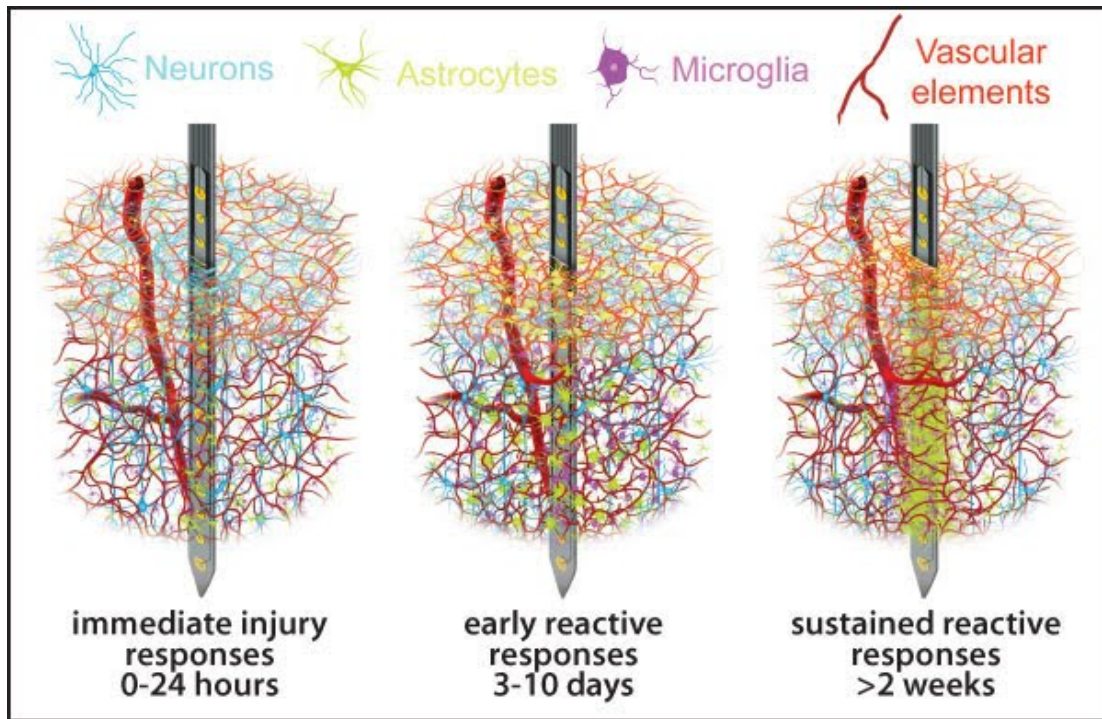


Figure 5. Foreign body response to the implanted multi-electrode array (<http://knol.google.com/k/neural-prosthesis-brain-interactions#>)

In comparison, the foreign body response/reaction to an implant in soft tissue is composed of foreign-body giant cells (FBGCs) and the components of granulation tissue (e. g., macrophages, fibroblasts, and capillaries), which is the specialized type of tissue considered as the hallmark of healing inflammation [41]. The composition of the foreign body reaction is determined by the chemical, physical, and topographical properties of the implant. After implantation, a sequence of events involved in inflammatory (acute and chronic) and wound-healing responses lead to foreign-body giant cells formation [41, 42]. Following inflammatory cell infiltration, monocyte adhesion, macrophage differentiation, and macrophage mannose receptor upregulation, the formation of foreign-body giant cells (FBGCs) is completed by the fusion of monocytes and macrophages in an attempt to phagocytose the implant material. Th2 helper lymphocytes is very

important in chronic inflammatory phase with the production of cytokines (IL (interleukin)-4 and IL-13), which can mediate monocyte/macrophage fusion to form FBGCs. Fibrosis surrounds implant with its interfacial foreign body reaction, isolating the implant and foreign body reaction from the local tissue environment. Also, the foreign body response may persist at the tissue/implant interface as long as the implant remains in soft tissue [41].

1.3 Current strategies and clinical demands

1.3.1 Development of microelectrode array technology

The general goal of developing neuron-electronic device interfaces for neural recording or stimulation is targeting particular areas of the nervous system at desired selectivity, fidelity, and functional duration. In the past 50 years, three generations of microelectrode arrays have been extensively studied as neuron-electronic device interfaces. The first chronic implantable MEAs were microwires [43]. In general, they are made of stainless steel or tungsten with an insulation layer of Teflon or polyimide. The key issues in their clinical applications as cortical neural prosthetics (CNP) are the uncertainty of optimal insertion depth and the change of the electrode tip's relative position in the brain, resulting from shrinking or expanding of the brain [1]. "Michigan arrays" and "Utah arrays" are representatives of the second generation: silicon substrate MEAs [1, 44, 45]. In contrast to microwires, as a result of multiple electrode sites deposited along the probe, "Michigan arrays" can be used at desired cortical depths for good extracellular recording [46], and their semiflexible ribbon cables allow them to move as the cortex pulses. Whereas the design of "Utah arrays" allows them to "float" on the cortical surfaces too, it has the advantage of providing a relatively large number of

electrode sites in a small package [47]. However, “Utah arrays” have the same “uncertainty of optimal insertion depth” problem as microwires. Instead of silicon substrates, MEAs of third generation have neuron-device interfaces based on biocompatible polymeric substrates, such as polyimide [48]. Polymers are promising candidates to provide ideal electrode surfaces for the selective attachment of various biomolecules, promoting long-term integration of tissue and device.

1.3.2 Investigations on electrode geometry and implantation techniques

Although the tissue reaction following electrode implantation is still not fully understood; the size and shape of the electrode and its insertion technique are primarily responsible for the damage imparted [27, 33, 49]. Since electrodes of a cylindrical shape can push blood vessels and cells away with minimal damage, they have been used in deep brain stimulation (DBS). Alternatively, a conical electrode shape with a sharp tip has been applied in most microwires [50, 51] and “Utah arrays” [52, 53]. The advantage of this design is that most adjacent tissue can remain intact after electrode insertion. However, the forces generated by the displacement/ compression of the surrounding tissue due to the presence of implanted electrodes will propagate through the tissue, leading to tearing, stretching, and compression of nearby tissue. In contrast, electrodes with a flat, sword-like tip have been used in a variety of “Michigan arrays” for their potential in minimizing surrounding tissue compression [54].

Investigation of electrode geometry and insertion techniques demonstrated that the size and shape of the electrode only affects the tissue reaction in the first 1-2 weeks post-implantation [10]. Except for the affected tissue volume, insignificant histological

difference was shown in the long run. Surprisingly, insertion techniques appeared to have little impact on the following tissue response [10].

1.3.3 Haptotactic and chemotactic cues for electrode biocompatibility improvement

Besides the modification of the electrode material, electrode geometry, and implantation techniques, haptotactic (e.g., electrode coatings [55-58]) and chemotactic cues (e.g., neurotrophic factors [57, 59, 60], anti-inflammatory drugs [61, 62] and adhesion molecules [56, 63, 64]) have been applied to enhance the neuron-electronic device interface. In particular, combination strategies that employ various cues have shown great potential in interface biocompatibility improvement.

To obtain robust and reliable chronic electrode performance, electrode surfaces have been modified using hydrogels, silk-like polymers, and nanotubes [65] in the past decade. Martin's group has successfully demonstrated that conducting polymers such as poly(pyrrole) (PPy) and poly(3,4-ethylenedioxythiophene) (PEDOT) can be electrochemically polymerized in template nanostructures (e.g., films, interconnected microcavities [66], microfibrils [67] and nanotubes (NTs) [65]) on neural electrode sites. In addition to decreasing impedance and increasing charge capacity, these patterned conducting polymer substrates can serve as drug carriers and release drugs through electrical stimulation. Shain *et al.* showed that the application of dexamethasone (DEX) could effectively reduce the density of the perielectrode sheath by inhibiting astrocyte hyperplasia [62]. To further improve neural interfaces, Martin's group combined electrode coatings with anti-inflammatory drug release (e.g., DEX), fabricating multifunctional substrates comprised of DEX-incorporated PLGA biodegradable

nanofibers, an alginate hydrogel layer, conducting polymer PEDOT nanotubes, and cloudy-like PEDOT inside the alginate hydrogel for the electrode/tissue interface [61]. Although the alginate hydrogel layer in the multifunctional substrates can reduce the initial burst effect of DEX to some degree, more work is needed to obtain a near-linear release profile.

Apart from anti-inflammatory drugs, controlled release of neurotrophins has also been employed to attract growing neurites and increase tissue/electrode integration. For example, poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogel-coated substrates were developed and assessed for storage capacity and NGF delivery in the brain by Jhaveri *et al.* [60]. While the results confirmed that pHEMA hydrogel-coated devices loaded with NGF can be made to stimulate neurite extension in the first 5 days, the problem caused by chronic reactive response near the neural interfaces wasn't solved. Poly(ethylene glycol) (PEG) based hydrogels have also been studied as electrode coatings. Winter *et al.* investigated the neurotrophin-eluting ability of poly(ethylene glycol)- poly(lactic acid) (PEGPLA) hydrogel electrode coatings [57]. Similarly, although promising, modifications are necessary to reduce the initial release burst and increase neurotrophin release duration.

Another important strategy is the use of adhesion molecules. The incorporation of adhesion molecules can increase the interaction between the device and neural tissue by promoting cell adhesion and controlling cell migration. Laminin peptide YIGSR molecules have been added to electrodes, greatly enhancing cell adhesion [64]. Bellamkonda *et al.* examined neural electrode coatings consisting of polyethylimine (PEI) / laminin, PEI/ gelatin/ chitosan/ gelatin, and PEI/ gelatin, which promoted neuronal

adhesion and neurite extension *in vitro* [56] and reduced early microglia activation over a period of 4 weeks *in vivo* [63].

In sum, electrode coatings with the potential to attract neurites to the electrode surface, control neuron adhesion and migration, and reduce the formation of the encapsulating sheath are required to address the problem of electrode biocompatibility. Additionally, when electrode coatings act as drug carriers, they should be tailored to provide an effective biomolecule release at a desired therapeutic dose and duration (over 8 weeks to reduce the glial scar formation).

To address these challenges, we constructed hydrogel-electrospun fiber mat composites to provide a “cell friendly” environment. In contrast to the existing coating strategies discussed above, our hydrogel composite materials not only have the potential to lower acute/early immune response by providing biomolecule (neurotrophins) and guidance (nano-topographical properties for cell adhesion) cues, but also can be modified to release biomolecules over 2-months in a near-linear way to reduce chronic immune response.

1.4 Dissertation overview

This dissertation is divided into 7 chapters. In **Chapter 2**, hydrogels and electrospun fibrous scaffolds, the major components utilized in composite construction, are described. Hydrogels, the crosslinked form of hydrophilic polymers, have demonstrated great potential for biomedical applications. Based on the polymers’ derivation and composition, hydrogels can be characterized as synthetic, biological, or hybrid biomaterials. For a particular application, hydrogel properties (e.g., 3D structure, mechanical strength and modulus, chemical and biological properties) can be tailored by

modulating the hydrogel's design and synthesis processes. Additionally, a variety of responsive hydrogels have been investigated, sensing and responding to the change of environmental stimuli, such as temperature, pH, and specific analytes. In the past couple of decades, hydrogels have been widely applied in tissue engineering, controlled drug delivery, and diagnostic devices such as biosensors and microarrays.

Electrospun fibrous scaffolds can be made of a variety of natural, synthetic, or hybrid polymers, through the use of different electrospinning techniques. The morphology and architecture of electrospun fibrous scaffolds can be tailored to mimic the structure of native extracellular matrix (ECM), making electrospun fibrous scaffolds great candidates for tissue engineering applications. In addition, due to their high area-to-volume ratio, electrospun fibrous scaffolds have been extensively investigated as controlled drug delivery systems. Also, general or specific bio-functional capability can be added into the electrospun fibrous scaffolds by incorporating biological cues during electrospinning or through post-electrospinning modifications. In this chapter, we summarize some applications of hydrogels and electrospun fibrous scaffolds in neural engineering. Finally, we discuss our proposed hydrogel-electrospun fiber mat composite materials.

Chapter 3 presents the syntheses of diacryl-PEG-polyester block copolymers and PCL-based electrospun fiber mats (EFMs). We characterized the intermediates and final diacryl-products of PEGPCL and PEGPLA copolymers using Fourier transform infrared spectroscopy (Nicolet 6700 FT-IR spectrometer) and Thermo Scientific nuclear magnetic resonance spectroscopy (^1H NMR Bruker DPX400). To alter PCL EFM hydrophobicity, we produced fluorinated PCL and acrylic acid (AAc) treated PCL EFMs through plasma

treatment. Also, the core-shell electrospinning technique was applied to fabricate PCL/PEGPCL core/shell EFMs. Water contact angles of different EFM samples were measured as an index of surface hydrophobicity by using a contact angle goniometer. EFM mean pore size and average fiber width were evaluated by analyzing their scanning electron micrographs. In addition, we measured EFM porosity through the ethanol displacement method.

In **Chapter 4**, we construct PEGPCL hydrogel-PCL EFM composite materials in two different configurations (A&B). Composite material release kinetics were studied using bovine serum albumin (BSA) as model protein. The analysis of BSA release profiles from both composite materials (A&B) demonstrated that when hydrophobic EFMs were applied as external layers covering the hydrogel layer (composite B), longer protein release duration and smaller initial burst release could be achieved. We further investigated the aggregation status of BSA released from composite B, and the efficacy of composite B *in vitro* using a PC12 cell line model. The results were promising, showing that composite B could not only extend protein release, but also retain protein bioactivity. Additionally, no significant cytotoxicity was found when PC12 cells were cultured in the presence of composite B.

Then, we fabricated PEGPLA hydrogel-EFM composite materials (composite B) using EFMs of different thicknesses (300 μm , 800 μm and 1100 μm) and hydrophobicity (water contact angle ranging from $\sim 0^\circ$ to $\sim 150^\circ$). The swelling and release behaviors of these composite materials were investigated, confirming our hypotheses that both EFM hydrophobicity and thickness have important impacts on the swelling and release

behaviors of composite materials, and can be modulated to control release rate, linearity, and burst release.

Chapter 5 explored the effect of our composite materials on cell adhesion using cell culture models of SK-N-SH cells and rat cortical cells. In SK-N-SH cell culture study, we seeded cells on top of six different samples: PEG hydrogels, PEGPCL hydrogels, PCL EFMs, PCL/PEGPCL core/shell EFMs, PEGPCL hydrogel-PCL EFM composite materials, and PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite materials. The results of both MTT cell adhesion tests and fluorescent cell viability studies demonstrated that cell adhesion capacity followed the trend of EFMs > composite materials > PEG-based hydrogels. In addition, there was no significant difference of cell adhesion capacity between samples in each category (hydrogel, EFM, or composite material). The morphology of SK-N-SH cells cultured on EFMs and composite materials was observed using scanning electron microscopy (SEM), and the analysis of cell spreading area and circularity showed that cells cultured on top of EFMs had smaller circularity than those cultured on composite materials. Similarly, rat cortical cells were cultured on top of composite materials: PEGPCL hydrogel-PCL EFM composite and PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite materials for 5 days. RNCs showed better interaction with the composites comprising of more hydrophilic EFM (PCL/PEGPCL core/shell EFM) than the composites with hydrophobic EFM (PCL EFM), confirming our hypothesis that scaffold's hydrophilicity played an important role in cell adhesion, especially for the cells which were sensitive to changes of the outside environment.

In **Chapter 6**, PEGPCL hydrogel-PCL EFM composite materials were applied as neurotrophin-eluting coatings for neural prostheses (multi-electrode arrays). We first investigated electrode coating adhesion ability through implantation studies, phosphate buffered saline (PBS) immersion tests, and agarose gel phantom tests that mimicked brain tissue constraint under *in vivo* conditions. The results confirmed coating adhesion capacity. In particular, the presence of external EFMs physically restrained the hydrogel layer, reduced the hydrogel degradation rate, and improved coating adhesion ability. Then, we incorporated nerve growth factor (NGF) into PEGPCL hydrogel-PCL EFM coatings and studied NGF elution using the NGF E_{Max} ELISA assay. The results showed that the composite coating was capable of delivering NGF at effective concentrations for over 3 weeks. In PC12 cell culture studies, we demonstrated that our electrode coatings could provide bioactive NGF for at least 2 weeks to stimulate neurite extension.

In **Chapter 7**, we summarize our results, make conclusions, and list our recommendations for future work.

Chapter 2: Hydrogels, Electrospun Fibrous Scaffolds and Their Applications in Neural Engineering

In the past couples of decades, a variety of biomaterials with the desired functional properties have been engineered to interface with biological systems. For clinical applications, biomaterials should be capable of interacting with the targeted tissues/cells and providing multiple physical, chemical, and biological cues which can act cooperatively and/or synergistically in the microenvironment [5, 68]. One class of biomaterials, hydrogels, the crosslinked forms of hydrophilic polymers, have been extensively explored and demonstrated their great potential in biomedical applications due to their high water content and biocompatibility [69]. To optimize their performance, hydrogels with various chemical structures, mechanical, biological and stimuli-responsive properties have been designed and widely applied as tissue engineering scaffolds and controlled drug delivery systems [37, 69-71]. Besides, hydrogels have also been integrated into microdevices, such as biosensors, microelectrode arrays and diagnostic imaging devices [5, 69].

A common strategy in tissue engineering to promote the tissue/material interaction is to mimic the architecture of natural ECM [70, 72]. It is believed that the topographical cues provided by ECM play an important role in the control of cell behaviors including cell adhesion, migration, proliferation and differentiation [73]. Thus, fibrous scaffolds have attracted a great deal of attention, and significant progress has been

made in designing, fabricating and using them for biomedical applications (e.g., drug delivery, artificial organs and tissue engineering) [72, 74]. Electrospinning is a simple technique to produce continuous fibers with diameters in the range of several nanometers to microns [75, 76]. As a close imitation of the ECM, electrospun fiber mats have the potential to provide a more conducive environment for cellular functions [72]. Additionally, micro- or nanofibers exhibit extremely high surface area-to-volume ratio, not only increasing cell/material contact area, but also enhancing drug uptake efficacy as drug carriers [77-79].

In this chapter, we briefly reviewed the rational design of hydrogel and electrospun fibrous scaffold, including the selection of polymer material and fabrication technique. In addition, we summarized some applications of hydrogels and electrospun fibrous scaffolds in neural engineering.

2.1 Hydrogels

Hydrogels are appealing candidates for biomedical applications because of their high affinity of water and 3D network structures [69, 70, 80]. Despite the widespread use of hydrogels in tissue engineering and drug delivery, there is an increasing need to develop novel hydrogels with functional properties desired in particular applications [69].

2.1.1 Hydrogel structure, design and material selection

As biomedical materials, the bulk structures of hydrogels primarily determine the hydrogels' suitability and performance in a particular application [69]. To characterize the network structures of hydrogels, equilibrium-swelling theory and rubber-elasticity theory have been widely applied in the measurement of three important structural

parameters [69, 81]: the polymer volume fraction in the swollen state ($v_{2,s}$, also known as hydrogel volumetric swelling ratio), the molecular weight of the polymer chain between crosslinking points ($\overline{M}_c$) and the corresponding mesh size (ξ) (Figure 6) [68].

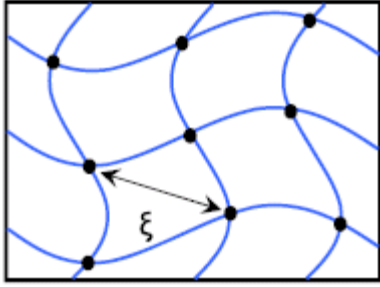


Figure 6. Simplified schematic of crosslinked hydrogel structure (black dots: cross-linking points; ξ : mesh size of the hydrogel) (Modified from [68]).

According to the Flory-Rehner theory [81], for hydrogels that do not have ionic moieties, they are only subject to the thermodynamic force of mixing and the retractive force of the polymer chains when immersed in a solution. At equilibrium, the two opposing forces are equal. The situation can be expressed in terms of Gibbs free energy:

$$\Delta G_{total} = \Delta G_{elastic} + \Delta G_{mixing} \quad \text{Eq. 2.1}$$

Here, ΔG_{total} , $\Delta G_{elastic}$ and ΔG_{mixing} represent the total Gibbs free energy, the Gibbs free energy resulted from the elastic retractive force of polymer chains, and the energy due to the spontaneous mixing of the surrounding solution molecules with polymer chains, respectively. For hydrogels that contain ionic moieties, an additional contribution to the total Gibbs free energy is the result of the ionic nature of the polymer network, ΔG_{ionic} . And thus, the expression of the physical situation should be modified as [69, 81]:

$$\Delta G_{total} = \Delta G_{elastic} + \Delta G_{mixing} + \Delta G_{ionic} \quad \text{Eq. 2.2}$$

After a series of derivations and the application of the rubber-elasticity theory, the resulting expression for determining $\overline{M}_c$ of a neutral hydrogel prepared in the presence of water can be written as:

$$\frac{1}{\overline{M}_c} = \frac{2}{\overline{M}_n} - \frac{\left(\frac{\bar{v}}{V_1}\right)[\ln(1-v_{2,s}) + v_{2,s} + \chi_1 v_{2,s}^2]}{v_{2,r}[(\frac{v_{2,s}}{v_{2,r}})^{1/3} - (\frac{v_{2,s}}{2v_{2,r}})]} \quad \text{Eq. 2.3}$$

Here, $\overline{M}_n$ is the molecular weight of the polymer chains prepared under the identical conditions without the presence of crosslinkers. $\bar{v}$ is the specific volume of the polymer, V_1 is the molar volume of water, χ_1 is the polymer-solvent interaction parameter, and $v_{2,r}$ is the polymer volume fraction in the relaxed state, which is after gelling but before swelling. The following equations 2.4 and 2.5 are expressions for anionic and cationic hydrogels prepared in the presence of a solvent, respectively:

$$\frac{V_1}{4IM_r} \left(\frac{v_{2,s}}{v}\right)^2 \left(\frac{K_a}{10^{-pH} + K_a}\right)^2 = [\ln(1-v_{2,s}) + v_{2,s} + \chi_1 v_{2,s}^2] + \left(\frac{V_1}{v\overline{M}_c}\right) \left(1 - \frac{2\overline{M}_c}{\overline{M}_n}\right) v_{2,r} \left[\left(\frac{v_{2,s}}{v_{2,r}}\right)^{1/3} - \left(\frac{v_{2,s}}{2v_{2,r}}\right)\right] \quad \text{Eq. 2.4}$$

$$\frac{V_1}{4IM_r} \left(\frac{v_{2,s}}{v}\right)^2 \left(\frac{K_a}{10^{pH-14} + K_a}\right)^2 = [\ln(1-v_{2,s}) + v_{2,s} + \chi_1 v_{2,s}^2] + \left(\frac{V_1}{v\overline{M}_c}\right) \left(1 - \frac{2\overline{M}_c}{\overline{M}_n}\right) v_{2,r} \left[\left(\frac{v_{2,s}}{v_{2,r}}\right)^{1/3} - \left(\frac{v_{2,s}}{2v_{2,r}}\right)\right] \quad \text{Eq. 2.5}$$

Rubber-elasticity theory can also be applied in the calculation of corresponding mesh size (ξ). ξ can be written as:

$$\xi = v_{2,s}^{-1/3} \left(\frac{2C_n \overline{M}_c}{M_r}\right)^{1/2} l \quad \text{Eq. 2.6}$$

Here, C_n is the Flory characteristic ratio, M_r is the molecular weight of the repeating units in the polymer chain, and l is the bond's length along the backbone of the polymer.

The molecular architecture of hydrogels can be easily modified to render desired mechanical, responsive and diffusive properties, rationalizing the numerous uses of hydrogels in biological and medical applications. While covalent crosslinks can form stable hydrogels, other types of crosslinks (e.g., hydrogen bonding, ionic bonding and formation of crystallites) can be added to control the hydrogels' gelling properties under some specific conditions (e.g., pH, pI , temperature and biological recognition) [69]. Depending upon their derivation and composition, hydrogels can be characterized as synthetic, natural or hybrid materials.

2.1.1.1 Synthetic hydrogels

Various polymerization methods can be applied in the design and synthesis of polymer network [68, 69]. As improvements continue to be made in the molecular-scale control over hydrophilic polymers, a variety of hydrogels with tailored properties (e.g., biodegradability [82, 83], mechanical strength [84], response to environmental stimuli [85-87]) have been synthesized and used in therapeutics. The most widely explored and used neutral synthetic hydrogels are made of poly(ethylene glycol) (PEG), poly(hydroxyethyl methacrylate) (HEMA), poly(vinyl alcohol) (PVA) and their derivatives (Figure 7) [69].

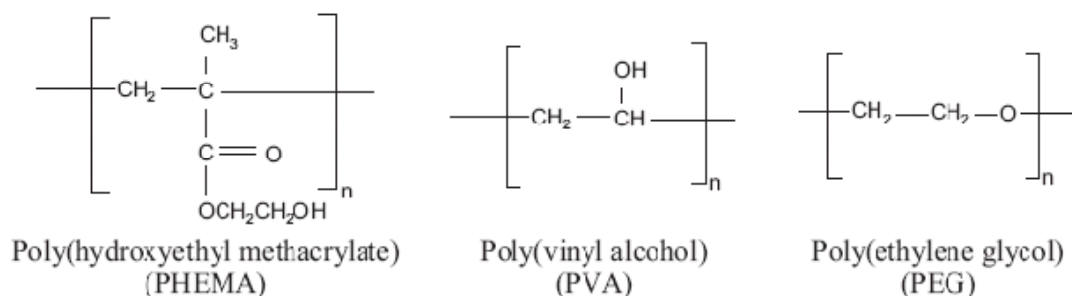


Figure 7. Chemical structures of PEG, PHEMA and PVA (Modified from [69])

PEG hydrogels have been approved by the US Food and Drug Administration (FDA) for various clinical applications, because they are non-toxic, non-immunogenic and biocompatible [69]. Since PEG, also known as poly(ethylene oxide) (PEO), can resist the adsorption of most biological molecules; it is often used as a “stealth material” [88]. Commonly, PEG hydrogels are applied as platforms, which can render a surface with desired properties such as the selectivity of specific biomolecules after PEG surface modification (e.g., covalent bonding through acrylate, silane, and thiol linkages, adsorption, ionic and hydrogen bonding) [89, 90]. To improve cell/material interaction, PEG gels can be modified through various conjugations, including the incorporation of adhesion molecules (e.g., peptide sequence) into gels [91]. To induce specific properties such as biodegradability, block copolymer of PEG (e.g., PEG-poly(lactic acid) (PLA) triblock copolymer [82]) can be synthesized in addition to chemical modification. Based on the types of PEG monomer’s functional groups and their average number, PEG hydrogels can be formed through chain-growth, step-growth, or mix-mode step and chain growth polymerization [68].

PHEMA is another hydrogel that has been widely studied and used for their mechanical properties, optical transparency and its stability in water [69]. PHEMA gels can be molded into various shapes and their mechanical strength can be increased by

adding multiple PHEMA layers in the scaffold [92]. PHEMA and its derivative, poly(hydroxyethyl methacrylate-co-methyl methacrylate) (PHEMA-MMA) are not degradable, making it possible to remain stable after implantation [93-95]. Similar to PEG, PHEMA can be modified to generate specific properties. For example, its degradation properties can be modulated by conjugating dextran with PHEMA gels [96].

As another major synthetic polymer, PVA hydrogels are stable and elastic, being formed by either physical (through repeating freezing and thawing procedures [97]) or chemical crosslinking processes. One of the commonly used crosslinking methods is the use of difunctional crosslinking agents (e.g., glutaraldehyde, acetaldehyde, formaldehyde, and etc.) [69, 98]. However, the difficulty of removing crosslinking agent residual from PVA gels makes it challenging for biomedical applications. Thus, PVA have been crosslinked through electron-beam or gamma irradiation to avoid the removal step of crosslinking agent residual [99, 100]. Additionally, PVA polymer can be modified and crosslinked through photo-polymerization [101, 102].

Recently, environmental sensitive hydrogels have attracted a great deal of attention for their ability to interact with their environment in a pre-programmed and intelligent manner [103]. The sensing and responding mechanisms are based on the molecular structure of the polymer network. For examples, hydrogels made of ionic polymers such as poly(acrylic acid), poly(methacrylic acid), polyacrylamide (PAAm), poly(diethylaminoethyl methacrylate), and poly(dimethylaminoethyl methacrylate), contain either weakly acidic or basic pendent groups [85, 104, 105]. The ionic gels' degree of swelling at equilibrium, changes as the solution pH and ionic composition

change. Here, the gels act as semi-permeable membranes, modulating the osmotic balance between the hydrogel and the surrounding solution through ion exchange [69].

As temperature is an important environmental parameter, temperature-responsive hydrogels, mostly made of poly(N-isopropylacrylamide) (PNIPAAm) and its derivatives, have been extensively studied and applied in drug delivery and tissue engineering [86] [103]. The hydrogels networks can shrink or swell reversibly as the environmental temperature is raised above or lowered below the polymer's lower critical solution temperature (LCST), below which the polymer is miscible in all proportions [69].

To control the molecular-recognition properties of hydrogels, it is desirable to organize the chemical structure in a precise 3D configuration. Thus, template-mediated polymerization techniques such as molecular imprinting [106], have been applied in the preparation of the recognition domains in hydrogels' 3D structure, greatly improving the system's efficacy at targeting specific molecules and demonstrating their great potentials for various biological analytes and physiological processes [107-109].

2.1.1.2 Natural hydrogels

In contrast to synthetic hydrogels, hydrogels from natural sources have low toxicity, mostly gel under mild or benign conditions, and can be degraded through natural means by specific proteinases [69]. In general, natural polymers' properties and utilities are determined by their origin and composition.

As the main protein of the mammalian extracellular matrix (ECM), collagen, as well as other derived protein-based polymers, is a great scaffold candidate for cellular growth [69]. And the cell behavior can be modulated through the presence of many cell-signaling domains in ECM. However, unlike most synthetic hydrogels, collagen

hydrogels can't provide sufficient mechanical strength in many situations. Thus, various methods (e.g., chemical crosslinking [110, 111], crosslinking with UV or temperature [110, 112], mixing with other polymers [110, 113]) have been developed to enhance the mechanical properties of collagen hydrogels.

Hyaluronic acid (HA), another natural polymer derived from the mammalian ECM component, is a linear glycosaminoglycan (GAG) with repeating disaccharide units. Almost found in all animal tissues, HA is an attractive choice for promoting wound healing and tissue repair [114]. In addition, HA is non-immunogenic, and contain some domains for cell adhesion. Because HA is water soluble, to obtain covalently crosslinked HA hydrogels, it is necessary to apply chemical modifications [115-118].

Agarose and alginate are linear polysaccharides, naturally derived from seaweed and algae respectively. While agarose gels can be formed through crosslinking with temperature; alginate scaffolds are formed by divalent cations (e.g., Ca^{2+}) crosslinking. Both agarose and alginate gels' mechanical properties largely depend on the gels' crosslinking densities, which can be modified by changing the monomer units and molecular weight of the polymer. Additionally, the mechanical properties of the gels will alter with time due to the slow degradation of the gels [69].

Chitosan, naturally derived from chitin, is another linear polysaccharide. Chitosan can be crosslinked through various means, including dissolving in a nonsolvent [119], or photo-crosslinking [120]. With respect to its pH sensitive nature, injectable versions of chitosan scaffolds have been developed, forming gels at neutral pH [121, 122]. Moreover, specific functionality can be introduced into chitosan gels using chemical modifications, making chitosan gels effective scaffolds for many biomedical applications [123, 124].

2.1.1.3 Hybrid hydrogels

Hybrid hydrogels, the integration of biological elements with synthetic hydrogels, can synergistically combine the desired biological properties (e.g., cell adhesion, specificity of binding, high affinity and etc.) with tunable physical and chemical properties (e.g., mechanical strength, external stimuli-responsive abilities) [69]. In the past, enzymes and genetically engineered proteins have been immobilized or incorporated into the network structures of hydrogels, generating stimuli-responsive gels which can shrink or swell in a pre-programmed manner [125-127]. Besides, some gels exhibit the ability in gating and controlled delivery of active biomolecules [126].

A common strategy in the syntheses of biohybrid materials is to modify hydrogels by adding some functional modalities which can dramatically enhance cellular adhesion [91, 128]. Conjugated biomaterials that are composed of amino acid sequences (e.g., RGD, GHK, YIGSR or IKVAV) and hydrogels (e.g., PEG or PVA hydrogels) have been studied for the improvement of cell adhesion [128-132].

In addition, degradable PEG hydrogels can be developed through the incorporation of proteases [133]. Also, the degradability of other hydrogels can be rendered by the conjugation of degradable units. For example, dextran-PHEMA hydrogels have been synthesized, exhibiting great enzymatic degradability [134].

Another kind of biohybrid hydrogels is made of growth factors and hydrogels. Instead of physical entrapment, growth factors can be covalently conjugated with the hydrogels, regulating cellular responses [135, 136]. For example, transforming growth factor beta (TGF- β) has been covalently attached to PEG hydrogels to control smooth muscle cell behavior [135].

2.1.2 Hydrogels in neural engineering

Natural, synthetic and biohybrid hydrogels have great utility for a variety of biomedical applications, ranging from tissue engineering to diagnostic devices, which have been reported in many reviews [37, 68, 69]. Here, the applications of acrylate-based polymers (e.g., poly(ethylene glycol) diacrylate (PEGDA), 2-hydroxyethyl methacrylate (HEMA) and their derivatives) in neural engineering will be discussed in this section.

2.1.2.1 Hydrogels' scaffold architecture in neural engineering

As described in Chapter 1, nerve regeneration greatly depends on the synergistic effect of haptotactic (e.g., contact guidance) and chemotactic cues (e.g., neurotrophic factors, adhesion molecules and cells). Thus, biomimetic hydrogels with proper modifications such as 3D patterning and biomolecular functionalization, are promising candidates to enhance nerve regeneration.

First of all, the interactions between neural cells and polymeric surfaces can be tailored through surface modifications including silane-based chemistries [137], electrostatic interactions [138], and plasma treatment [139]. Using these techniques, multiple biomolecules have been patterned on hydrogels without significant loss of bioactivity, improving the biocompatibility of hydrogel polymeric surfaces. However, several limitations have also been found, compromising the long-term application of these techniques. For example, it has been demonstrated that the use of the silane-treated surfaces will result in an increase of the hydrophobicity of hydrogel surface, decrease of fibronectin's affinity to its cell surface receptor, increased potential of cell detachment, and alterations in the cellular gene expression level [140-142]. Also, plasma treatment

may trigger undesirable reactions, leading to production of toxic by-products and deactivation of grafted biomolecules [37].

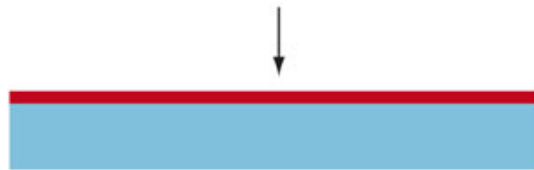
Since acrylate-based hydrogels are typical protein-resistant scaffolds, exhibiting minimal, non-specific cell binding capacity; it is critical to incorporate cell-adhesive cues into hydrogel templates for enhancing cell attachment, growth and proliferation [143, 144]. The most widely utilized techniques to pattern cells and biomolecules in hydrogel scaffolds include photolithography [145-147] and “soft” lithographic approaches (e.g., microcontact printing (μ CP) [148-150] and microfluidic patterning [151, 152]). Using a variety of photomasks, hydrogels with different shapes and 3D microstructures can be fabricated through photolithography [153, 154]. One advantage of photolithography is its capacity to create high density arrays with 3D microstructures on various micron-scaled substrates [155].

Microcontact printing (μ CP) provides a unique way to patterning biomolecules onto a surface by lift-off or casting method (Figure 8), where an elastomeric (e.g., polydimethylsiloxane) stamp, is used to deposit desired biomolecules into a specific pattern on a surface [156]. Previously, multiple biomolecules have been patterned into polyacrylamide (pAAm) hydrogels using μ CP [148, 157]. Another important “soft” lithography technique is microfluidic patterning, in which a substrate is coated with a hydrogel solution and then molded by a patterned elastomeric stamp. The resulting substrates typically contain two regions: a cell- and protein-resistant layer (molded hydrogel surface) and a cell- and protein-absorption region (exposed substrate surface). Microfluidic patterning has been successfully applied to pattern 3D hydrogels containing cells of multiple phenotypes [158, 159].

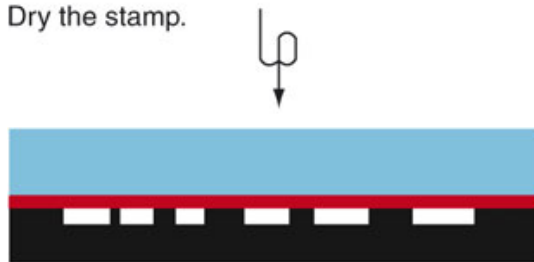
Lift-off method



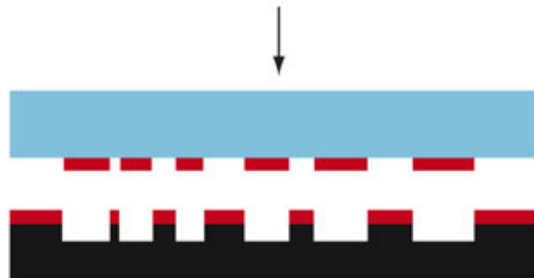
Cover the stamp with protein solution.



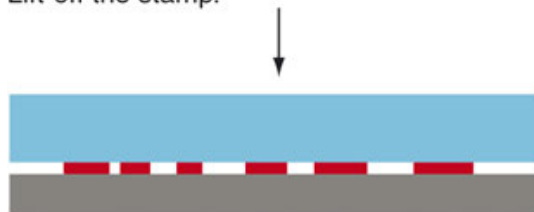
Dry the stamp.



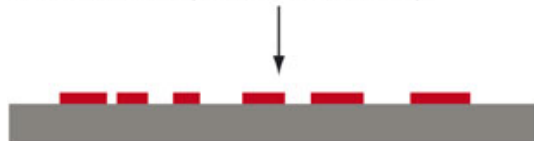
Place the stamp onto the silicon master.



Lift-off the stamp.



Place the stamp onto the coverslip.

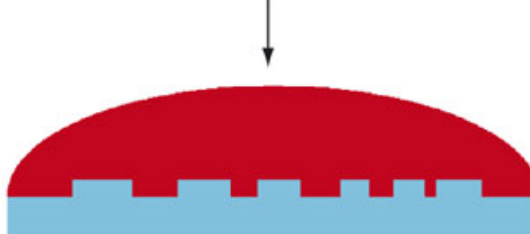


Complete pattern.

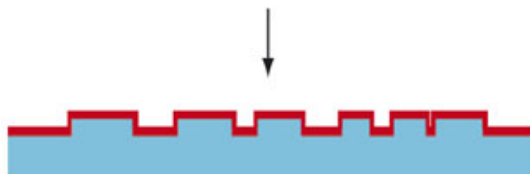
Casting method



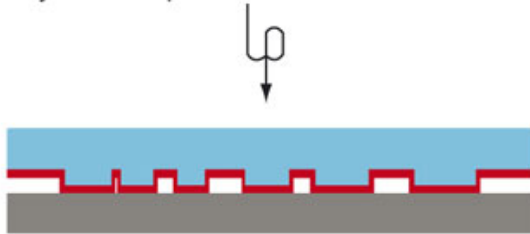
Cast a silicone replica from the silicon master.



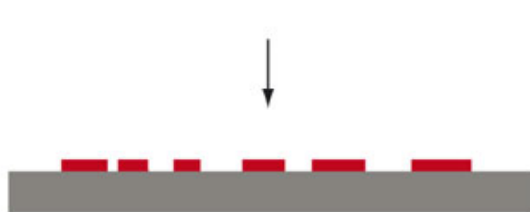
Cover the stamp with protein solution.



Dry the stamp.



Place the stamp onto the coverslip.



Complete pattern.

Figure 8. Microcontact printing [156]

The encapsulation of specific mammalian cell types within hydrogels has been studied for the treatment of neural disorders such as Parkinson's disease. The encapsulated cells potentially act as a physical barrier against a host foreign body response after scaffold implantation [160]. Mahoney and Anseth have demonstrated that when encapsulated inside a degradable 3D PEG hydrogel, neural cells can survive, proliferate, and differentiate into neurons and glia [161]. In addition, after proteolytically degradable peptide sequences being polymerized into PEGDA polymer backbone, cells can migrate through the polymer matrix as hydrogel is degraded by cell secreted proteolytic enzymes [162-164]. Importantly, the cross-linking density of hydrogels has been shown to alter cell metabolism, cell morphology and cellular gene expression [165, 166]. Dodla and Bellamkonda have created anisotropic 3D hydrogel scaffolds with photo-immobilized gradients of laminin-1 to enhance neurite extension *in vitro* [167]. The results showed that the anisotropic scaffold could accelerate the neurite extension from dorsal root ganglia (DRG) cells, obtaining a maximal growth rate twice of that in control groups (isotropic hydrogels of laminin-1). And, it is widely acknowledged that chemotaxis has important influence on dictating cell migration [168].

Also, a variety of strategies have been applied to generate macroporous PEG hydrogels for neural tissue engineering applications [169, 170]. For example, through a combination of photo-polymerization and a foaming process, highly interconnected macroporous PEG hydrogels can be created [170]. Important parameters such as pore dimension and swelling rate can be tailored by polymer concentrations as well as bubbling agent composition in the macromer solution. Ford *et al.* have crosslinked PEG with poly(L-lysine) around salt-leached poly(lactic-co-glycolic) acid (PLGA) scaffold,

and applied the resulted hydrogel in the co-culture of neural progenitor and endothelial cells *in vivo* [169]. The open macroporous hydrogel network appeared to support the formation of tubular vascular structures by brain endothelial cells, indicating the efficacy of macroporous PEG hydrogels as tissue-engineering scaffolds.

3D hydrogels guidance channels/ tubes, incorporated with biological cues (ECM proteins, cells, or growth factors), have been applied to promote axonal regeneration within the spinal cord. For example, Tsai et al. have fabricated poly(HEMA-co-methyl methacrylate) (HEMA-MMA) hydrogel guidance channels to facilitate axonal regeneration [94, 95]. Either seeded with neural tissue [95] or grafted with ECM components [94], HEMA-MMA hydrogel channels were found to significantly improve nerve regeneration after complete spinal cord transection. Additionally, coil-reinforced hydrogel tubes have been developed, exhibiting the same potential to promote nerve regeneration as nerve autografts [93, 171, 172].

2.1.2.2 Controlled delivery of biomolecules from hydrogels in neural engineering

Besides the incorporation of cells and adhesion molecules into hydrogels systems, the delivery of neurotrophic factors and anti-inflammatory drugs has great potential for the applications in the CNS. In general, drugs can be loaded into hydrogels through entrapment, tethering, or the incorporation of micro- or nanoparticles, as depicted in Figure 9 [68]. For example, the entrapment of drugs can be achieved by *in situ* encapsulation (drugs are dispersed in macromer solution before hydrogel cross-linking) or post-fabrication equilibrium partitioning (gels are incubated in concentrated drug solution for drug loading) (Figure 9A). Also, drugs (e.g., growth factors) can be tethered into hydrogels through enzyme-sensitive peptide linkers (Figure 9B). These gels are

attractive candidates for tissue regeneration applications, owing to their ability to degrade and release active growth factors as migrating cells secrete specific proteolytic enzymes [173-175].

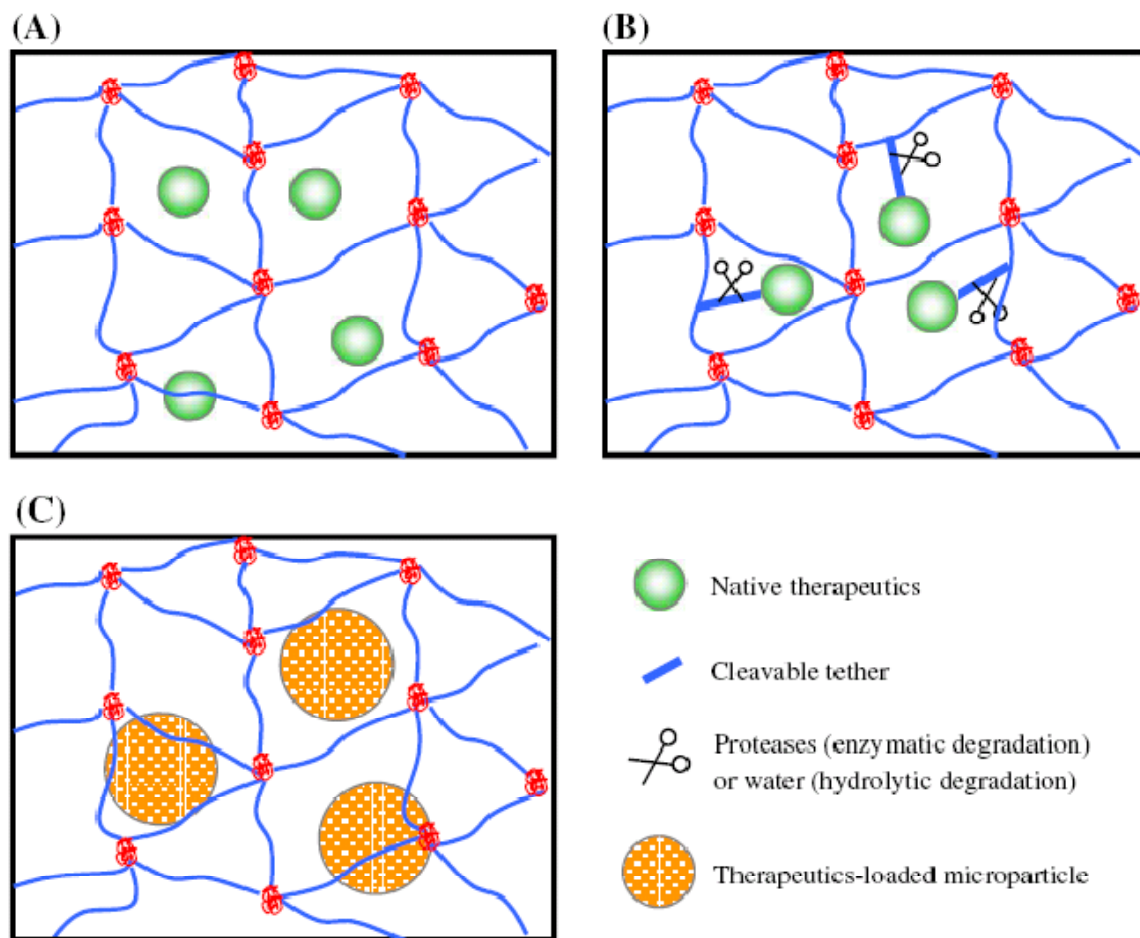


Figure 9. Methods for therapeutics loading into PEG hydrogels ([68])

In addition, the use of micro- or nanoparticles offers a unique way to incorporate therapeutics into hydrogel network structures (Figure 9C). Multiple drugs can be loaded into bulk hydrogel and dispersed micro- or nanoparticles simultaneously [176, 177]. And the delivery rates of drugs encapsulated in different phases can be modulated separately by adjusting the parameters that would affect hydrogel (e.g., hydrogel cross-linking

density) or microparticles properties (e.g., polymer material) [177]. Burdick *et al.* [178] and Mikos *et al.* [177] have individually synthesized such composite hydrogel systems for dual growth factor delivery. Their works demonstrated the hydrogel-microparticles scaffolds' excellent capacity of providing multiple drugs at different release rates. In particular, the use of the combination of hydrogel and microparticles phases significantly reduced the burst effect (obtained relatively high drug concentration at the initial release stage) in the release profiles of factors entrapped inside microparticles, and extended their release durations [178].

Hydrogel-based controlled release systems can be divided into five categories (Figure 10) [69]: A) non-swollen, non-degradable, non-stimuli-responsive hydrogels (drugs are uniformly dispersed inside hydrogel network structure, having a release rate primarily determined by diffusion process); B) core-shell structure hydrogels (drugs are retained in the core, being encapsulated by hydrogel shell); C) swollen hydrogels (the hydrogel swelling increases the gel's mesh size in the swollen region, and accelerates the release rate of drugs dispersed outside the rubber core); D) enviro-stimuli-responsive hydrogels (hydrogels can sense and respond to the changes of external stimuli such as temperature, pH value, and ion strength, leading to the release of encapsulated drugs); E) degradable hydrogels (the drug release rate relies on both diffusion and gel degradation processes).

In the past, controlled drug release from polymeric acrylate-based hydrogels has been extensively studied, showing that the drug release rate is dependent on a variety of factors, including monomer type, utilized cross-linker, cross-linking density, the molecular weight of drug, drug loading percentage, the pH value of medium and so on

[179-181]. For example, Burdick *et al.* have applied photo-polymerized PEG-based hydrogels to deliver neurotrophins for the enhancement of neurite extension [129]. They found that the neurotrophins could be released without significant compromise of the protein bioactivity, and the release durations could be tuned from weeks to several months by adjusting the hydrogel cross-linking density.

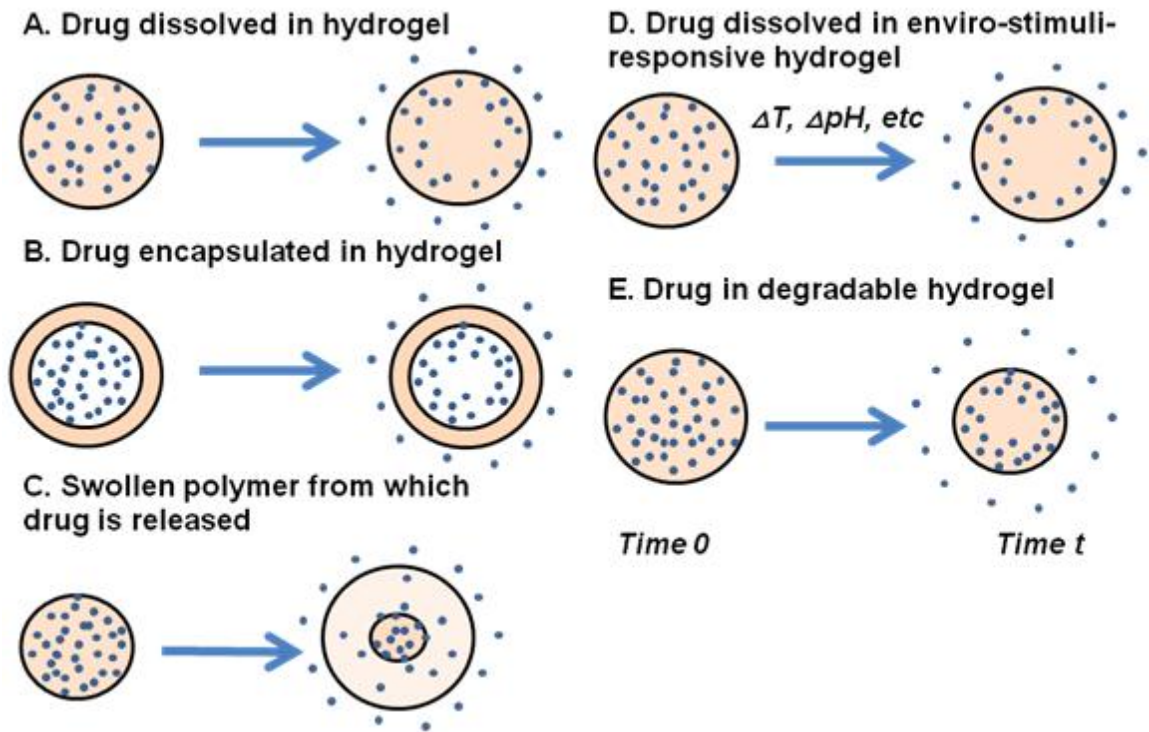


Figure 10. Hydrogel-based controlled release systems. (Redrawn from [69])

2.2 Electrospun fibrous scaffolds

Electrospinning is a simple and unique technique of producing non-woven fibrous articles. The electrospun fibers can be formed out of a variety of materials including natural and synthetic polymers, composites and ceramics, with the fiber diameters ranging from tens of nanometers to microns, which is otherwise difficult to be achieved

by conventional non-woven fiber fabrication methods [182, 183]. Many parameters contribute on the manipulation of architecture and morphology of the resulting fibrous scaffold, including polymer solution properties (e.g., viscosity, elasticity, surface tension and conductivity), electrical field strength, field pattern, temperature, humidity, and etc [184]. In the past several years, various innovative electrospinning techniques have been developed to introduce desired functions and properties to the electrospun fibers for biomedical engineering applications [72].

2.2.1 Rational design of electrospun fibrous scaffolds

As well as the architecture and morphology, the physical and biological properties of the electrospun fibrous scaffolds, such as hydrophilicity, biocompatibility, biodegradability, mechanical strength, and specific cell/scaffold interaction, play an important role in their biomedical applications. Since these physical and biological properties are largely determined by the materials' chemical composition, the success of the electrospun fibrous scaffolds' application in a particular situation depends on the material's selection to a large extend [72]. In addition to taking advantage of materials chemical compositions, by selecting a suitable fabrication process, which modulates the scaffolds' morphology and architecture (e.g., porosity, fiber diameter, and scaffold's thickness), electrospun scaffolds can be further tailored with desired functions [72].

2.2.1.1 Natural, synthetic and hybrid polymers

In the past, all four major classes of natural polymers: proteins, polysaccharides, DNA, and lipids, have been electrospun into fibrous scaffolds. Protein fibers, formed out of collagen, elastin, gelatin and silk fibroin (SF), have been fabricated and applied in biomedical engineering [185-187]. For example, while collagen fiber scaffolds

demonstrated their great potential in enhancing cell growth and infiltration [188, 189]; SF electrospun fibers have become a good candidate for wound dressing and tissue engineering due to their ability to promote cell adhesion and proliferation [190-192]. Recently, polysaccharides, such as HA, dextran, chitosan (chitin) and cellulose acetate, have also fabricated. In particular, HA, which used to be considered as a difficult-to-process polymer in terms of its high solution viscosity, has been processed through “blowing-assisted” electrospinning, and become a promising candidate for cartilage repair [193, 194]. Although the fibers made of DNA or lipid, have not been explored as extensively as those of protein or polysaccharide; some researchers reported the feasibility of the fabrication of DNA or lipid nanofibers (e.g., calf thymus Na-DNA [195] and lecithin [196]), implicating their potential applications in controlled drug delivery and tissue engineering.

Compared with natural occurring polymers, though exhibiting less specific cell/material interaction, synthetic polymers usually offer many advantages such as cheaper prices and predictable batch-to-batch uniformity [72, 197]. Also, synthetic polymers can be tailored to provide a wider range of physical and chemical properties. Typical electrospun synthetic polymers used in tissue engineering are hydrophobic biodegradable polyesters (e.g., polyglycolide (PGA) [198, 199], polylactide (PLA) [200, 201], poly(ϵ -caprolactone) (PCL) [202, 203] and poly (3-hydroxybutyrate) (PHB)) [197]. On the other hand, electrospun fibers formed out of some hydrophilic biodegradable polymers, such as polyurethane [204], poly(vinyl alcohol) (PVA) [205, 206], PEO [207], polydioxanone [208] and polyphosphazene derivatives [209], have also been widely used in biomedical applications .

In addition, when properly fabricated, synthetic copolymers can be used in a wider range of biomedical applications with significantly improved properties compared to homopolymers. For example, copolymers of biodegradable hydrophobic polyesters (e.g., poly(lactide-glycolic acid) (PLGA) [210, 211] and poly(lactic acid-caprolactone) P(LA-CL) [212, 213]) have been extensively studied with respect to their great mechanical properties. By selecting different chemical compositions and adjusting the composition ratio, the scaffolds' mechanical properties [212] and degradation rates [213] can be manipulated for specific applications. Besides biodegradability, the hydrophilicity of the electrospun scaffolds can be modulated by adding a hydrophilic polymer block (e.g., PEG, PVA and etc.) into the copolymer [214, 215]. DegraPol[®], a degradable polyester-urethane block copolymer, has been electrospun into fibrous scaffolds and applied in skeletal muscle tissue engineering, exhibiting great cell affinity as well as processibility and elasticity [216].

Polymer blending provides a straightforward means to combine different polymers for biomedical applications [72, 217]. Unlike the syntheses of block copolymers, polymer blending is easier and less expensive, due to the bypass of complicated copolymer synthetic schemes. For example, collagen and elastin have been blended and electrospun into fibrous scaffolds that appeared to be suitable as viable vascular tissue engineered constructs [217]. As natural polymers often have weak mechanical properties while synthetic polymers are usually lack of cell affinity, the advantages of blending natural and synthetic polymers are evident. Electrospun scaffolds based on the blends of natural and synthetic polymers, combine the great durability, mechanical strength and modulus of synthetic polymers with the specific biological

affinity of natural polymers, demonstrating their attractive potentials in biomedical engineering [72]. One example is the gelatin/PCL nanofibrous scaffolds, which were found to be readily able to support the attachment, growth and infiltration of bone-marrow stromal cells (BMSC) [218]. Heparin and PEG have also been mixed and electrospun into nanofibrous scaffolds, which can significantly prolong the release of heparin, suggesting their potential use in wound dressings and controlled drug delivery [219]. In addition, scaffolds based on the blends of type I collagen/PEG [220] or wool keratin/PEG [221] were also fabricated and applied in biomedical engineering.

As a hydrophobic copolymer which possesses great mechanical properties, PLGA has been widely blended with other polymers, especially hydrophilic polymers (e.g., dextran [222], PEG-g-chitosan [223], PEG-PLA copolymers [224] and etc.). With the enhanced hydrophilicity, tunable degradability and sometimes specific cell affinity, these PLGA-based electrospun scaffolds can be used as tissue engineering constructs or drug-carriers with controlled-release capability. In contrast, PEG/PEO, a unique hydrophilic and amphiphilic polyether diol, has also been frequently mixed with other polymer for electrospun scaffold fabrication. Besides improving the fiber properties (e.g., hydrophilicity), the addition of PEG/PEO can also facilitate the electrospinning of some difficult-to-process biomaterials [223, 225, 226].

2.2.1.2 Novel fabrication techniques for electrospun fibrous scaffolds

Besides using the appropriate material for electropinning, the application of a suitable fabrication technique is needed for creating the desirable 3D architecture and surface morphology of the micro- or nanofibrous scaffolds. In tissue engineering, scaffolds with properly aligned fibers are often desired in certain applications for guiding

cell growth and migration [186, 227-229]. The aligned fiber collection methods include auxiliary electrode/electrical field [230-232], thin wheel with sharp edge collector [233], frame collector [184], and mechanical drawing [234]. Among all of these methods, mechanical drawing (e.g., uniaxial drawing and sequential biaxial drawing) is probably the most practical method. Human coronary artery smooth muscle cells (SMCs) have been seeded and cultured on the aligned electrospun scaffolds that were fabricated by the use of a rotating disk collector with a sharp edge, exhibiting improved cell adhesion and proliferation rate [228]. In addition, the cytoskeleton proteins' distribution and orientation were found to be parallel to the direction of nanofibers. The fiber orientation can also be obtained by a post-drawing process after electrospinning. And the aligned fibrous scaffolds have been used to promote the growth of cardiac myocytes (CM), demonstrating their potential in the manipulation of cardiac tissue structure and functions [235].

By depositing several individual electrospun fiber layers sequentially on the same target collector, composite scaffolds of different polymers and even different mesh sizes can be achieved. This technique is termed multilayer electrospinning [236]. The use of the composite scaffolds has been demonstrated in the studies of bone regeneration [237] and biohemostat [238]. Scaffolds composed of two different polymers can also be fabricated through mixing electrospinning technique, having two different polymer solutions electrospun simultaneously from different syringes and under different process conditions [236]. Also, nanofibrous scaffold based on PLA/clay nanoparticles has been produced, where the salt leaching/gas forming process generated micro-sized pores inside

the scaffold. The co-presence of the micro-sized pores and nano-sized pores formed by the entanglement of fibers made it a unique dual-porosity electrospun scaffold [239].

Immiscible polymer solutions can be electrospun too from a single syringe, forming fibers with a distinct two-phase structure. The two-phase electrospinning technique provides an effective means to incorporate biomolecules into the electrospun mats, showing great potential in the applications of controlled drug delivery and tissue engineering [240]. Another frequently used fabrication technique for controlled drug release applications is core-shelled electrospinning [241]. Using this method, several difficult-to-process materials could be processed as the core within the shell of other polymers [242]. PCL shell has been used to protect the dextran core containing bovine serum albumin (BSA) and prolong the BSA release [243]. Moreover, poly(dimethylsiloxane) (PDMS) has been selected to form the shell, protecting the living cells suspended inside the core from the possible cellular damage resulted from the fabrication process [244]. Additionally, blowing-assisted electrospinning technique has demonstrated its potential in assisting the electrospinning of polymer materials with high solution viscosity and high surface tension [193, 194]. The air blowing process showed many advantages, such as decreasing the polymer solution viscosity by elevating the blown air's temperature, accelerating the solvent evaporation, and modulating the resulting fiber diameters by controlling the air temperature and flow rate [193].

To further improve the physical and biological properties of the electrospun scaffolds, surface modifications are often required after electrospinning. The commonly employed methods include plasma treatment [245-247], wet chemical method [248, 249], and surface graft polymerization [250, 251]. For example, PCL mats with increased

hydrophobicity can be produced by plasma treatment in a CF₄ atmosphere [252]; whereas more hydrophilic PCL mats can be generated by acrylic acid (AAc) graft polymerization after argon plasma treatment [253]. Moreover, the following immobilization of collagen on the PCL-AAc mats can further enhance cell attachment and proliferation rate [253]. Also, poly(L-lactic acid)-co-poly(ϵ -caprolactone) (P(LLA-CL)) electrospun scaffolds have been coated with collagen and used to promote the attachment, spreading, viability of the human coronary artery endothelial cells [254]. Gelatin was grafted onto the surface of the electrospun poly(ethylene terephthalate) (PET) scaffold to overcome the biological inertness of the surface and induce specific cell affinity [255].

2.2.2 Applications of electrospun fibrous scaffolds in neural engineering

Since electrospun fibers can be structured in various configurations, mimicking the architecture of native ECM [72]; electrospun scaffolds have found applications in different areas of tissue engineering ranging from cardiovascular [235], skin regeneration, musculoskeletal tissue engineering [216], to stem cell engineering [256] and neural engineering [227, 257]. The feasibility of applying electrospun fibrous scaffolds to enhance nerve regeneration has been investigated both *in vitro* and *in vivo*, demonstrating the fibers' great potential in the applications of neural engineering.

2.2.2.1 Effects of guidance cues of electrospun fibrous scaffolds on nerve regeneration

In general, 3D scaffolds used in neural engineering should be able to serve as contact guidance, providing topographical cues for neurite outgrowth and cell migration, two indispensable processes for nerve repair [258]. Both random and aligned electrospun fibrous scaffolds have been applied to stimulate nerve regeneration. Rat dorsal root

ganglia (DRG) explants were cultured on PLLA randomly-oriented and aligned nanofibers for 6 days [259]. The results showed that while no evident neurite outgrowth from DRG explants was found on randomly-oriented fibers, significant neurite extension was observed on aligned fibers. It also appeared that the aligned fibers directed the orientation of neurite outgrowth and cell alignment. Apparently, neurites extended in the direction of the aligned fibers grew much faster than those extended perpendicular to the aligned fibers' direction or those on randomly-oriented fibers. In addition, many studies have found the similar results that neurons cultured on aligned fibers exhibited longer neurite extension than those on randomly-oriented fibers [260-262].

Astrocytes have also been cultured on randomly-oriented nanofibers, displaying a branched network structure with no direction preference. In contrast, when cultured on aligned fibers, well-aligned astrocytic processes were observed, following the direction of the aligned nanofibers [262]. Additionally, ganglia were found to experience elongated morphological change when cultured on aligned fibers [261]. And the alignment of glial cells would further accelerate neurite extension [263]. Moreover, these morphological alterations may lead to functional changes that will affect cellular phenotypes, such as apoptosis, cell proliferation, migration, differentiation, contractility and gene expression [264-266].

In PNS, after nerve injuries, Schwann cells need to migrate and align to form bands of Büngner for the following axonal infiltration [267]. In order to enhance the migration and alignment of Schwann cells, blended PCL/collagen aligned fibrous scaffolds were used to guide the formation of bands of Büngner *in vitro* [260]. The results demonstrated the effectiveness of the aligned fibers on inducing Schwann cells migration

away from the DRG along the align fiber axis. Cells can even stretch their cytoskeleton across multiple fibers and migrate along the fiber direction to some extent, when cultured on random fibrous scaffolds.

To evaluate the efficacy of electrospun fibrous scaffolds in enhancing neural functional recovery, several *in vivo* studies have carried out in both CNS and PNS models. Random electrospun polyamide nanofibers have been used for over-hemisection spinal cord injury in rats [268]. Although axonal outgrowth was found at injury sites after 3 weeks, the regenerated axons couldn't target the distal stump by following the randomly-oriented fiber, suggesting the employment of multilayered tubular structure for axonal guidance. Panseri *et al.* applied PLGA/PCL electrospun fibrous tubes to bridge nerve gaps in the rat sciatic nerve [269]. The results of using random electrospun fibrous tubes for nerve gap bridging were promising. After 4 months, significant regeneration of axon and tissue was found, filling the fibrous tubes and bridging the nerve gaps. In addition, initial functional recovery was seen in the treated injured animal group, while no evident recovery was observed in the untreated injured animal group.

Kim *et al.* fabricated nerve regeneration guidance conduits by stacking several electrospun fiber sheets into polysulfone [270]. Both randomly-oriented and aligned fiber sheets were applied, respectively. In a rat sciatic nerve transection model, after 16 weeks, significantly higher number of regenerated axons per cross-section was found in the conduits comprising of the aligned fibers. Myelination and axonal extension into the distal end were only found in the aligned constructs. Accordingly, when grid walking test and electrophysiology test were carried out to evaluate functional recovery, similar reactions (e.g., muscle contraction and compound action potential (CAP)) were observed

in injured animals treated with autografts and conduits made of aligned fibers, whereas no CAP was detected in animals having nerve gaps bridged by random constructs.

Alternatively, hollow guidance conduits comprising of a rolled-up sheet of aligned electrospun fibers were fabricated and applied to bridge a 15 mm nerve gap in the rat sciatic nerve [271]. The inner surface of the conduits was composed of aligned fibers oriented either longitudinally or circumferentially, both providing large nerve cross-sectional area. Interestingly, no significant differences of nerve regeneration and functional recovery were found between the animals treated with longitudinally-oriented fiber conduits and those treated with circumferentially-oriented fiber conduits.

In general, most studies were limited to the evaluation of cell morphological changes and proliferation. More analysis is required to elucidate the functional changes (e.g., gene expression) of neural cells and the mechanisms, such as cell signaling processes, behind the cellular behavior corresponding to topographic stimuli.

2.2.2.2 Effects of biological cues of electrospun fibrous scaffolds on nerve regeneration

Besides guidance cues, electrospun fibrous scaffolds can serve as promising drug-carriers, releasing bioactive molecules (e.g., neurotrophic factors) in a controlled manner [197]. For example, human β nerve growth factor (NGF) has been incorporated into aligned electrospun fibers made of PCLEEP, a copolymer of caprolactone and ethyl ethylene phosphate by Chew *et al.* [272]. The protein can be uniformly distributed in electrospun fibers and sustained released for over 3 months *in vitro*. Using a rat sciatic nerve transection model, the same group has demonstrated that the human glial cell-derived neurotrophic factor (GDNF) -encapsulated aligned electrospun fibrous conduits

can significantly enhance nerve regeneration, when compared to the control group comprising of plain fibrous scaffolds [271].

In addition, biological cues can be introduced into electrospun fibrous scaffolds through surface modifications after electrospinning. Patel *et al.* have immobilized basic fibroblast growth factor (bFGF) onto electrospun nanofibers to stimulate DRG neurite extension [259]. As a comparison, they studied four different groups: plain align electrospun fibers, bFGF-immobilized randomly-oriented fibers, bFGF-immobilized aligned fibers, and a positive control having bFGF directly added into DRG culture medium. The highest neurite outgrowth was found in the groups of bFGF-immobilized aligned fibers and positive controls, indicating the great potential of the surface-modified aligned fibrous scaffolds as delivery vehicles.

Another important application of electrospun fibrous scaffolds is to deliver stem cells to the injured sites in the nervous system. Stem (progenitor) cells are multipotent, being able to differentiate into various cell lineages. Thus, the transplantation of stem cells can replace missing cells as well as activate endogenous cells for self-renewal [273]. To obtain deeper understanding of stem cell-scaffold interaction, various studies have been performed using CNS model. For example, neural stem cells (NSCs) have been cultured on both PLLA randomly-oriented and aligned electrospun fibers [227]. Interestingly, the results showed that the cell differentiation rate was largely determined by fiber diameter rather than fiber alignment. Also, NSCs cultured on aligned 300 nm diameter fibers exhibited significantly longer neurite extension than those on other groups, suggesting the promising application of nanoscale diameter aligned fibers in neural engineering.

Nisbet *et al.* increased the hydrophiliity of both PLLA and PLGA electrospun scaffolds by using potassium hydroxide [257]. As a result, scaffolds' surface tension was reduced, leading to an accelerated neurite outgrowth from the seeded murine embryonic cortical neurons. In this work, they also found that while neurons extended neurites along fiber direction when the fiber concentration was low, neurite extension crossed perpendicular to fibers in the regions with high fiber density. Thus, neurite outgrowth direction can be controlled by modulating fiber density in scaffolds.

In another work, mouse embryonic stem (ES) cells were cultured on electrospun nanofibers in the presence of retinoic acid, an essential chemical to induce ES cells differentiation along the neural lineage [274]. While both randomly-oriented and aligned fibrous scaffolds supported the ES cells differentiation into neurons, oligodendrocytes and astrocytes; it appeared that ES cells differentiation into astrocytes was discouraged on the aligned fibers. So, aligned fibers may be a promising approach to reduce the formation of glial scar, of which astrocytes are the primary component.

In addition to neurotrophic factors and stem cells, other biological cues such as adhesion molecules can be incorporated into electrospun fibrous scaffolds through a number of fabrication processes and post-electrospinning surface modifications, which have been discussed previously in Section 2.2.1. In a word, as multi-functional substrates that provide topographical and biochemical signaling simultaneously, electrospun fibrous scaffolds can serve as potential candidates for enhancing neural tissue regeneration.

2.3 Proposed hydrogel-electrospun fiber Mat (EFM) composite materials

The goal of our research is to construct a novel hydrogel-electrospun fiber mat (EFM) composite system, which combines the advantages of hydrogels and EFMs (as described previously in this chapter) into a single construct, designed to improve biocompatibility at the neuron-electrode interface. In addition, these materials may have great potential for tissue engineering and controlled drug delivery applications. In this study, we synthesized PEG-polyester diacrylate block copolymer with respect to their biocompatibility, biodegradability, and controlled drug delivery capacity. Because of the formation of PEG hydrogels through ultra violet (UV) photo-crosslinking, they can be formed into a variety of shapes and sizes and easily coupled with EFMs. PCL-based EFMs were fabricated as another integral component in composite material owing to their great mechanical properties and ability to provide suitable morphology and architecture to enhance tissue-scaffold interaction. Also, the hydrophobicity and cell affinity of PCL EFMs can be tuned by using core/shell electrospinning technique or post-electrospun modifications (e.g., plasma treatment).

In the aspect of controlled drug delivery, we anticipate that the incorporation of EFMs into hydrogel can provide additional diffusion barriers for the drugs dissolved in hydrogel phase. The increase of both diffusion path length and drug-scaffold interaction (hydrophobic/hydrophilic interaction) is probably the primary reason for the anticipated reduced initial burst release and extended release duration. In addition, the drug release profiles could be greatly altered by modifying composite material's configuration and the properties of either hydrogels or EFMs.

When constructed in some specific configurations (e.g., configurations with EFMs as external components), the morphology and surface properties of hydrogel-EFM composite materials may be determined by EFMs' properties rather than PEG hydrogels. In other words, these composite materials can provide more topographical cues for cell interaction (e.g., cell adhesion, migration and proliferation) than PEG hydrogels which are known for their resistance of protein absorption and cell adhesion.

Our hydrogel-EFM composite materials can be used as coatings for micro-devices such as MEAs for the enhancement of their long-term performance. When applied in neuroprosthetic devices (e.g., MEAs), the hydrogel-EFM coatings can provide sustained neurotrophin release, attracting the nearby neurons to extend their neurites towards the implanted devices, promoting the formation of a better interface between neurons and devices, and therefore counteracting some negative effect of acute and chronic inflammation on devices' long-term performance. Additionally, when properly designed, not only the controllable release ability and degradability of composite materials can be modulated, but also specific bio-functional capacities (e.g., cell affinity) can be introduced into composite materials, further enhancing the tissue/device interaction.

Besides the applications described above, our hydrogel-EFM composite materials are potential systems to provide dual release of biomolecules, capable of generating two or even more different release profiles simultaneously. Also, the great versatility of their configurations, as well as their ease processability, makes our composite materials promising candidates for a variety of tissue engineering applications.

2.4 Conclusions

Owing to their physical, chemical and biological properties, hydrogels have been widely applied in biomedical applications. Polymer engineers can design and modulate the hydrogels' properties through various synthesis approaches for a particular situation. In addition, electrospun fibrous scaffolds hold great potential in tissue engineering and controlled drug delivery for their ECM-resembled architecture and high area-to-volume ratio. The properties of electrospun fibrous scaffolds can also be tailored by the use of a variety of electrospinning techniques and post-electrospun modifications.

Our hydrogel-EFM composite materials combine the advantages of both hydrogel and EFMs, and therefore can be used as tissue engineering scaffolds or controlled drug release carriers. Our composite materials are anticipated to provide better cell-tissue interaction and generate pre-programmed drug release profile. Additionally, the properties of our composite materials can be tuned by the use of all the means suitable for the modulation of hydrogels or electrospun fibrous scaffolds. By applying our composite materials as neurotrophin-eluting coatings on neuroprosthetic devices such as MEAs, we expect to obtain a better interface between nearby neurons and MEAs, leading to a more reliable long-term electrode performance.

Chapter 3: Syntheses of Hydrogel-Electrospun Fiber Mat

Composite Component Materials¹

In Section 1.3, we explored current strategies for improvement in “biocompatibility” of the neuron-material interface. Among these approaches, coating the surfaces of electrodes with controlled release scaffolds has attracted much interest; as it holds great potential to incorporate biologically derived elements into microelectronics technology. The advantages of using biocompatible and biodegradable scaffolds for electrode surface coatings and drug delivery are *a)* reduced inflammatory response to prevent glial scar formation; *b)* promoting neuronal survival and functional recovery by providing a favorable environment, probably including both *cell adhesion* sites for neuron regeneration and *therapeutic drugs* for long term axonal extension; *c)* making it possible to remove the biodegradable scaffold after the chronic immune response and therefore have minimal negative effects on electrode function in the long run. To successfully design a scaffold for neural tissue engineering, scaffold material and structure, therapeutic drug, and drug delivery method should be taken into account.

Degradable PEG-based hydrogels have been widely studied as controlled release systems [3, 57, 69, 275-277] and tissue engineering constructs [69, 278]. Typically, these materials are composed of PEG hydrogels modified with degradable segments, of which the most popular are hydroxyacids (e.g., poly(lactic acid) (PLA) and poly(ϵ -caprolactone)

¹ Portions of this material have been submitted to Journal of Controlled Release and Journal of Biomedical Materials Research Part A.

(PCL)) [82]. The fundamental relationship between the structure of these materials and their swelling, mechanical, and release properties has been widely explored [80, 275]. The two primary concerns identified from these studies are the availability (delivered dosage) and stability (bioactivity) of released therapeutics [68], both of which can be enhanced through modification of polymer/ crosslinker/ initiator concentrations [279, 280] or introduction of stabilizing agents [276, 279]. However, despite significant progress in this field, non-linear release profiles with high initial release rates (i.e., burst release) are typical, especially for hydrophilic therapeutics with low molecular weight [281]. And burst release is highly undesirable because it can result in local or systemic toxicity, loss of therapeutic compounds, reduced therapeutic bioactivity, and shortened release duration[281].

One approach that has been successful in improving release kinetics is the combination of degradable PEG hydrogels with other release systems, such as micro- or nanoparticles [177, 178]. Inspired by this approach, we proposed to combine degradable PEG hydrogels with EFMs to alter release kinetics and obtain desired release profiles. EFMs have been extensively studied as tissue engineering constructs for their potential to topographically mimic the ECM, which is important in controlling cell adhesion, cell morphology, and tissue architecture [79, 188, 282]. Besides modifying therapeutic release profiles, the inclusion of degradable PCL EFMs promises to enhance cell attachment and therefore compensate the “stealth” or anti-fouling property of PEG hydrogels and promote tissue-device integration. Also, EFMs with high porosity and surface area are attractive candidates in controlled drug delivery [225] and are capable of maintaining the bioactivity of agents incorporated into fibers by electrospinning [272]. So, hydrogel-EFM

composite materials may be able to simultaneously deliver more than one kind of therapeutic with different release profiles.

In Chapter 3, we synthesized diacrylated poly(ethylene glycol)-poly(lactic acid) (PEGPLA) and poly(ethylene glycol)-poly(ϵ -caprolactone) (PEGPCL) block polymers, and characterized them with Fourier transform infrared spectroscopy (FT-IR spectrometer) and nuclear magnetic resonance spectroscopy (^1H NMR). Also, poly(ϵ -caprolactone) EFMs with different thicknesses and hydrophobicity were fabricated and evaluated using scanning electron microscopy (SEM).

3.1 Materials and methods

3.1.1 Syntheses of poly(ethylene glycol) (PEG)-based copolymers

3.1.1.1 Synthesis of diacryl- poly(ethylene glycol) - poly(ϵ -caprolactone) (PEGPCL) copolymers

PEGPCL copolymers were synthesized following a modification [277] of the method of Hubbell et al. [82]. Briefly, poly(ethylene glycol) (PEG) (MW 950-1,050) dried by azeotropic Dean-Stark distillation in anhydrous toluene was subjected to ring-opening polymerization in the presence of ϵ -caprolactone (2:1 molar ratio) and stannous octanoate (Tin(II)2-ethylhexanoate) catalyst at 130 °C under argon for 24 h. PEGPCL hydrogels with different numbers of ϵ -caprolactone (CL) repeats yield different degradation and drug release rates, with fewer CL repeats producing slower degradation and release rates. A ratio of ϵ -caprolactone to PEG of 2:1 was chosen to minimize degradation and release rates. Smaller ratios could not be employed because hydrogels with fewer caprolactone repeats are not degradable. It should be noted that no difficulties

in degradation were observed with our PEGPCL hydrogel, suggesting successful polymerization.

The resulting copolymer (PEGPCL) was cooled to room temperature, dissolved in anhydrous dichloromethane (CH_2Cl_2), added slowly to ice-cold hexane, collected by filtration, and dried overnight. The PEGPCL intermediate dried by azeotropic Dean-Stark distillation in anhydrous toluene was added to triethylamine (TEA, to neutralize HCl byproduct) and anhydrous dichloromethane. Acrylation was initiated by slow addition ($\sim 0.20\text{mL/min}$) of acryloyl chloride (ACCl) in anhydrous dichloromethane ($4M$ excess) at $-30\text{ }^\circ\text{C}$ for 8 h, and $25\text{ }^\circ\text{C}$ for 60 h. TEA-HCl salt was removed by repeated filtration, drying, and re-dissolution in anhydrous tetrahydrofuran. The final product was collected by dissolution in anhydrous dichloromethane, precipitation in ice-cold diethyl ether, and Buchner funnel filtration, and was dried in a vacuum oven in the presence of phosphorous pentoxide (P_2O_5). All chemicals were obtained from Sigma-Aldrich and used as received, unless otherwise stated.

3.1.1.2 Synthesis of diacryl- poly(ethylene glycol) - poly(lactic acid) (PEGPLA) copolymer

The procedures of diacryl-PEGPLA copolymer synthesis were published previously [57], modified from the method developed by Hubbel et al. [82]. Similar to the synthesis of diacryl- PEGPCL copolymer, poly-(ethylene glycol)-poly-lactic acid (PEGPLA) copolymer was synthesized through the overnight reaction between poly-(ethylene glycol) (PEG, molecular weight $M_w = 950\sim 1,050$) and lactide in a molar ratio of 1:2 at $120\text{ }^\circ\text{C}$ under argon atmosphere. PEGPLA was then diacrylated by AccI, and purified, following the same procedures used for diacryl-PEGPCL synthesis, except that the diacrylation time at $25\text{ }^\circ\text{C}$ was 40 h rather than 60 h.

3.1.1.3 Characterization of PEGPCL and PEGPLA copolymers

The intermediates and diacrylated copolymers of both PEGPCL and PEGPLA were characterized using Fourier transform infrared spectroscopy (Nicolet 6700 FT-IR spectrometer) and Thermo Scientific nuclear magnetic resonance spectroscopy (^1H NMR Bruker DPX400).

3.1.2 Fabrication of electrospun fiber mats

3.1.2.1 Fabrication of poly(ϵ -caprolactone) (PCL) electrospun fiber mats

A 12 wt% solution of poly(ϵ -caprolactone) (Sigma-Aldrich, Inc. M_w =65,000) in dichloromethane (Mallinckrodt Chemicals) was prepared by continuous stirring at room temperature. The solution was then placed in a 60 cc syringe with a 20 gauge blunt tip needle and electrospun using a high voltage DC power supply (Glassman High Voltage, Inc.) set to +30 kV, a 20 cm [283] tip-to-substrate distance, and a 3 mL/hr flow rate. A 3x3" (7.6 x7.6 cm) sheet of unaligned fibers was deposited onto aluminum foil. Fiber mats with different average thicknesses (300 μm , 800 μm , or 1100 μm) were produced by simply varying deposition time. The PCL sheets were then placed in a vacuum overnight [284] to ensure removal of residual dichloromethane. Various sized discs (diameters: 8 mm, 6 mm, or 1.5 mm) were then cut out of the nanofiber mat using dermal punches (Acuderm) for use in composite creation.

3.1.2.2 Fabrication of PCL/PEGPCL core/shell electrospun fiber mats

PCL/PEGPCL core/shell mats were synthesized as described above except that (a) an 18 wt% poly(ϵ -caprolactone) (PCL) in acetone solution and (b) a 50 wt% PEGPCL copolymer (synthesized as described previously [57, 277]) in acetone solution were used for the core and shell, respectively. Core shell fibers were prepared using a 22 gauge

hypodermic needle (Integrated Dispensing Solutions Agoura Hills, CA) inserted through a 16 gauge hypodermic T-junction (Small Parts, Inc. Miramar, FL) to create two concentric blunt needle openings. A Swagelok stainless steel union was used to hold the needles in place and ensure that the ends of the needles were flush with each other. One syringe (BD Luer-Lok tip) was filled with the polymer solution for the core, connected to the 22 gauge needle and set to a 10 mL/hr flow rate using a syringe pump. Another identical syringe was connected via an extension to the T-junction, filled with the shell material and set to a flow rate of 1 mL/hr using another syringe pump. Electrospinning was then carried out as described in Section 3.1.2.1.

3.1.2.3 Fabrication of fluorinated PCL electrospun fiber mats

Fluorinated PCL mats were produced by plasma treatment (Harrick Plasma) in a CF₄ atmosphere under vacuum of 1000 mT at a power of 30W for 1 minute.

3.1.2.4 Fabrication of acrylic acid-treated PCL electrospun fiber mats

Acrylic acid-treated PCL EFMs were produced via activation using an argon plasma pretreatment and subsequent acrylic acid (AAc) graft polymerization as described previously [253, 285]. Briefly, PCL fiber mats were placed in a quartz, cylindrical glow discharge cell (Model SP 100, Anatech Ltd., USA) filled with Ar and subjected to glow discharge at 37 W plasma power and 40 kHz radio frequency for 10 seconds. For graft polymerization, AAc solution (0.11 g/mL) and vitamin B2 (0.0152 mol/L) were mixed at a ratio of 1:20. Subsequent radical-initiated polymerization was inhibited by the addition of vitamin B2 because of the reduction of the dissolved oxygen.

The plasma treated PCL fiber mats were immersed in this solution and exposed to UV illumination for 30 min at room temperature. The modified mats were then washed

with doubly distilled water to remove residual AAc homopolymer, and dried in a vacuum desiccator.

3.1.2.5 Characterization of electrospun fiber mats

Water contact angles of all EFMs were measured at room temperature using a contact angle goniometer (Ramé-hart Instrument co., Model 200). Five measurements were carried out for each EFM. The morphologies of gold sputter-coated PCL, PCL/PEGPCL core/shell, fluorinated PCL, and PCL-AAc EFMs were examined by SEM (Quanta 200, FEI) at three magnifications ($N = 3$ for each magnification). SEM images were analyzed using Image J image analysis software (NIH) to measure average fiber width (ω) (at least 75 fibers/ image, $N = 3$), and mean pore size (at least 50 pores/ image, $N = 3$). EFM porosity (p) was evaluated using the ethanol displacement method [286]. Briefly, an EFM sample was immersed in ethanol with a known volume (V_1) and pressed several times to force the air, which filled the open pores of the EFM sample, out of the scaffold. The sample was then kept in ethanol for 10 min to permit ethanol penetration and displacement of air in open pores. The total volume of ethanol in the EFM sample was recorded as V_2 . After the removal of the ethanol-soaked EFM sample, the volume of the ethanol remaining was recorded as V_3 . Porosity can be calculated from the following equation [286]:

$$p = \frac{V_1 - V_3}{V_2 - V_3} \quad \text{Eq. 3.1}$$

One-way analysis of variance (ANOVA) with Tukey's test was applied to compare groups of data. A probability value of 95% ($p < 0.05$) was used to determine significant difference among groups. All data is reported as the mean $\pm$ the standard deviation.

3.2 Results and discussion

3.2.1 Characterization of poly(ethylene glycol) (PEG)-based copolymers

The chemical structure of diacryl-PEGPCL copolymer is shown in Figure 11. For the PEGPCL intermediate, notable peaks consisted of 1750 cm^{-1} = PCL, COO; 2870 = PEG, CH_2 ; and 3445 = PEG, OH for FT-IR (Figure 12). For the final acryl-PEGPCL product, peaks included 1730 cm^{-1} = acryl, COO; 1750 = PCL, COO; 2870 = PEG, CH_2 ; and 3445 = PEG, OH (absent or reduced dramatically because of the consumption of the terminal hydroxyl groups during diacrylation) for FT-IR (Figure 12) [287].

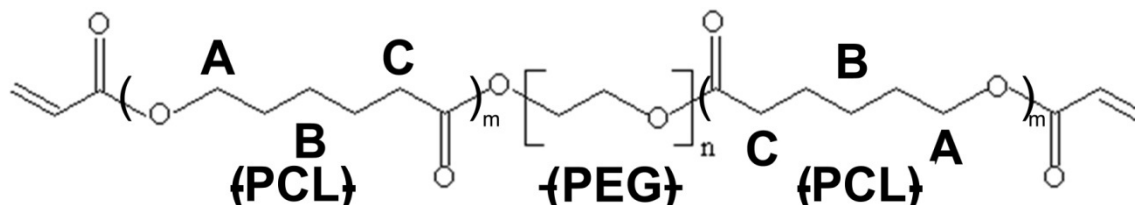


Figure 11. Chemical structure of diacryl-PEGPCL copolymer. Letters indicate significant chemical functional groups.

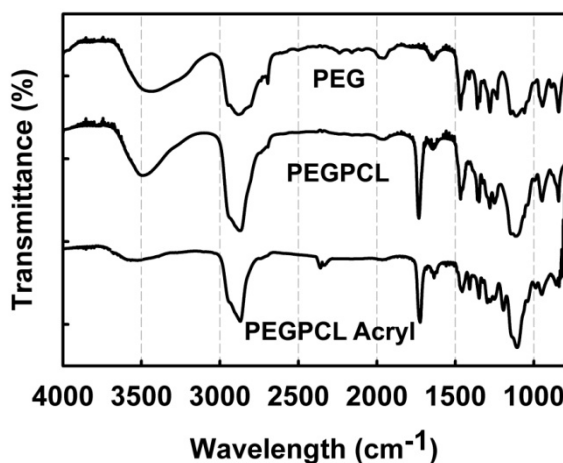


Figure 12. Representative infrared spectrum showing PEG, PEGPCL and diacryl-PEGPCL polymers.

For the final PEGPCL-diacrylated product, notable ^1H NMR peaks consisted of 3.64 ppm = PEG, CH_2 , 4.08, 4.15, 4.23, 4.31 = PCL, CH_2 (**A**, position as shown in PEGPCL chemical structure, Figure 12), 1.67 = PCL, CH_2 (**B**), 2.33 = PCL, CH_2 (**C**), and 5.81, 6.12, 6.41 = acryl, CH, CH_2 (Figure 13) [57, 287-289]. According to the ratio of integrated peak areas, the yields of PEGPCL intermediate and acryl-PEGPCL were 90.4% and 83.6%, respectively, consistent with previous results reported by our group [287].

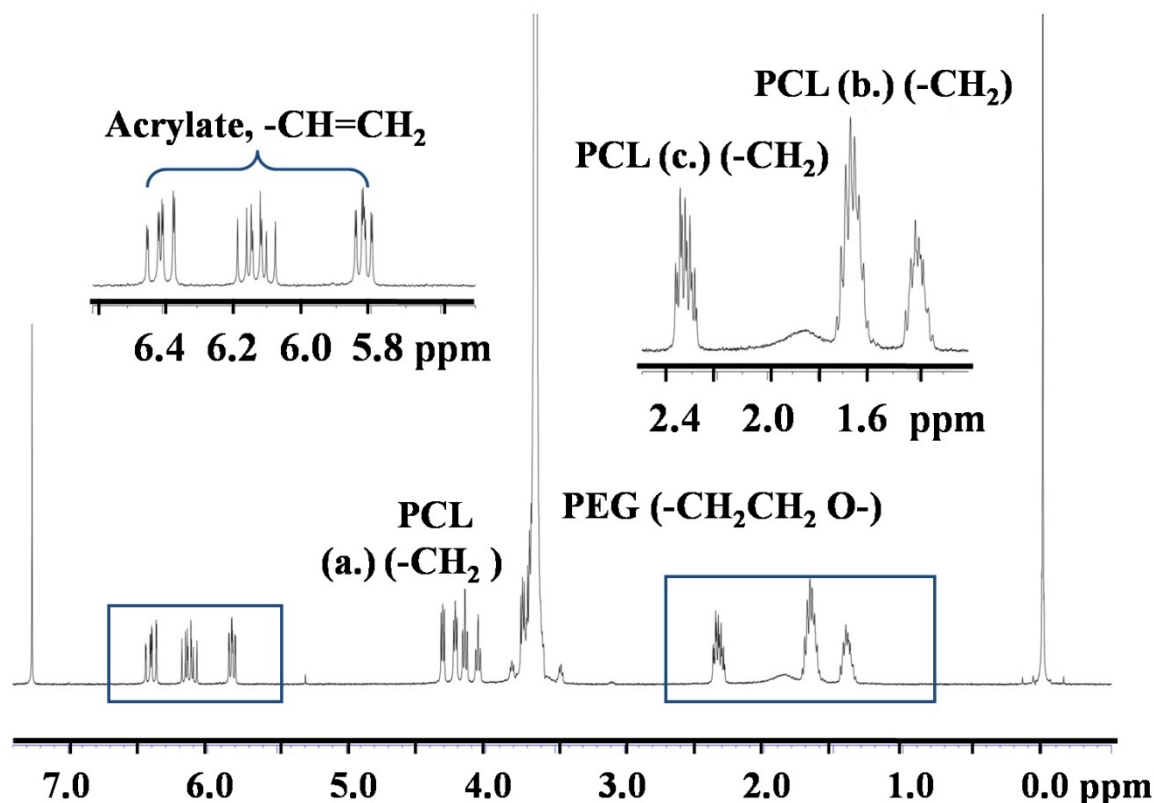


Figure 13. Representative ^1H NMR spectrum showing diacryl-PEGPCL copolymer

In addition, the chemical structure of diacryl-PEGPLA copolymer is shown in Figure 14. In FT-IR spectra (Figure 15), notable peaks consisted of 1750 cm^{-1} = PLA, COO; 2870 = PEG, CH_2 ; and 3445 = PEG, OH for the PEGPLA intermediate; and for the

final acryl-PEGPLA product, peaks from acrylate, lactide, and PEG were present at 1730 cm^{-1} = acryl, COO; 1750 = PLA, COO; 2870 = PEG, CH_2 ; and 3445 = PEG, OH (absent) [287].

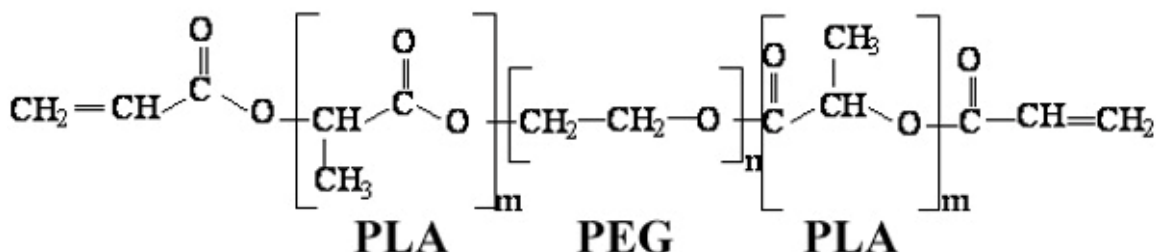


Figure 14. Chemical structure of diacryl-PEGPLA copolymer.

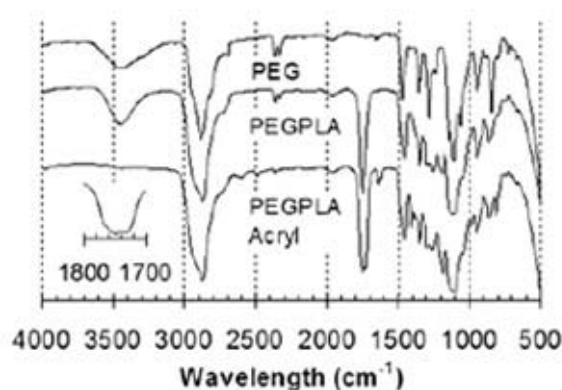


Figure 15. Representative infrared spectrum showing PEG, PEGPLA and diacryl-PEGPLA polymers [57].

For the final PEGPLA-diacrylated product, notable ^1H NMR peaks consisted of 1.55 = PLA, CH_3 , 3.64 ppm = PEG, CH_2 ; 4.92 = PLA, CH ; and 5.81 , 6.12 , 6.41 = acryl, CH , CH_2 (Figure 16) [57, 287-289]. The ratios of integrated peak areas represented the yields of PEGPLA intermediate and acryl-PEGPLA, which were 93.2% and 85.4%, respectively.

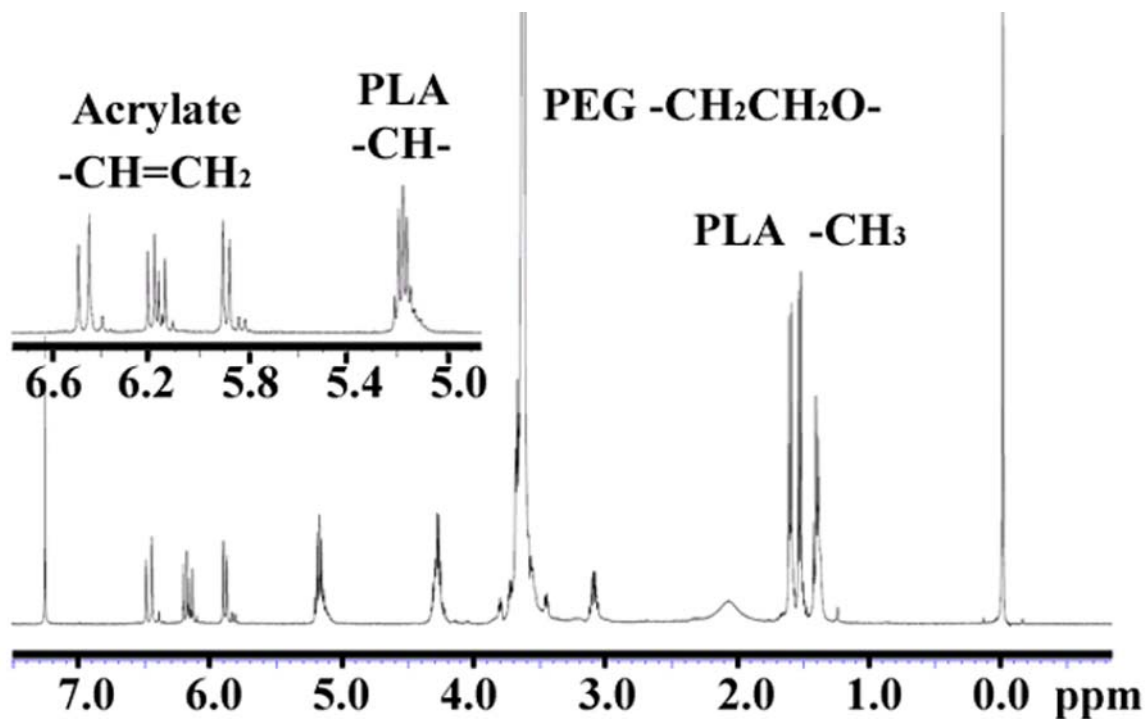


Figure 16. Representative ^1H NMR spectrum showing diacryl-PEGPLA copolymer

3.2.2 Characterization of electrospun fiber mats

To evaluate porosity, 300 μm , 800 μm , and 1100 μm thick PCL, 1100 μm thick PCL/PEGPCL core/shell, fluorinated PCL, and PCL-AAc EFMs (Figure 17), and thick PCL EFMs were characterized using scanning electron microscopy (SEM). No differences in pore size, structure, or uniformity were apparent. Additionally, average fiber width and mean pore size for each sample were determined (Table 1), and one-way ANOVA statistical analysis indicated no significant difference ($p > 0.05$) in these properties for all 6 types of EFMs investigated. Similarly, EFMs exhibited porosities, as measured by the ethanol displacement method, in the range of 0.75 to 0.83 with no significant difference ($p > 0.05$) between samples.

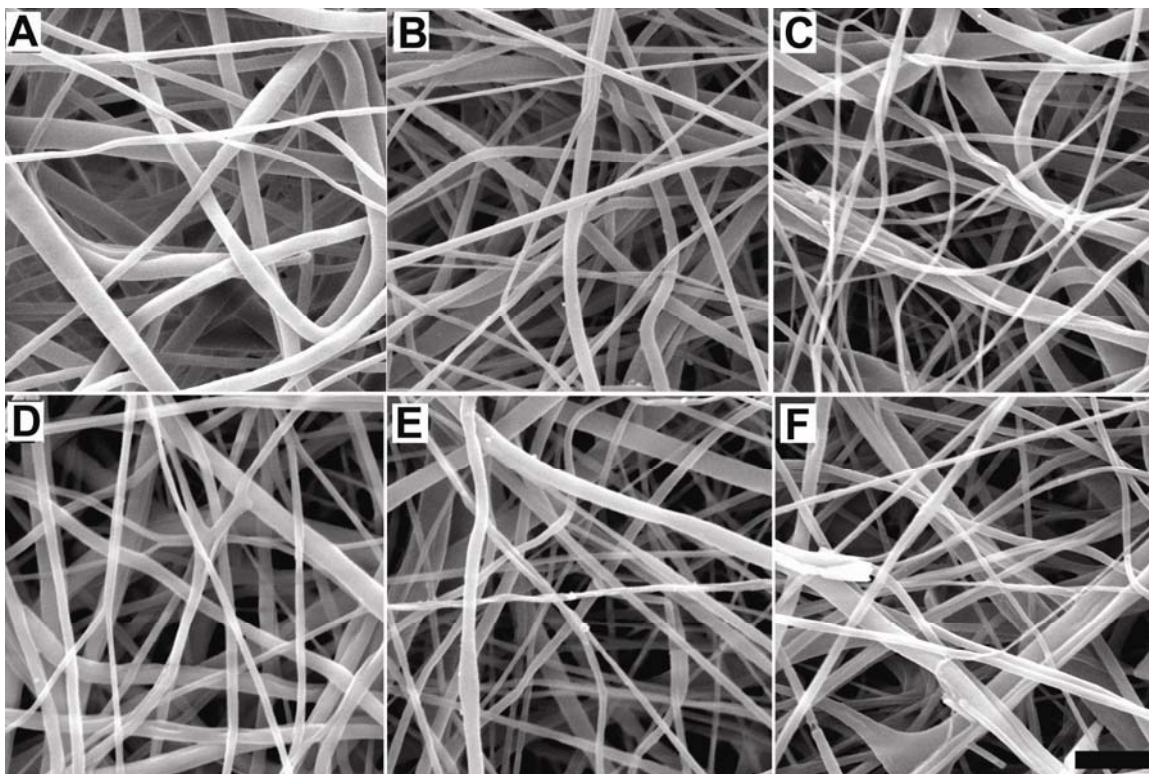


Figure 17. Representative scanning electron micrographs of different types of EFMs (scale bar: 10 μm) (A) 300 μm PCL; (B) 800 μm PCL; (C) 1100 μm PCL; (D) PCL/PEGPCL core/shell; (E) Fluorinated PCL; and (F) PCL-AAc EFMs.

EFM water contact angles were also evaluated (Table 1) and confirmed the effectiveness of EFM surface treatments as values were consistent with previous reports [253, 290, 291]. Average water contact angles for PCL EFMs increased slightly from 135.2° to 149.6° with thickness (300, 800 and 1100 μm). However, these changes were far less significant than those exhibited after surface treatment. Fluorinated PCL and PCL EFM contact angles (157.0° and 149.6°, respectively) were significantly higher than those of PCL/PEGPCL core/shell and PCL-AAc EFMs ($\sim 0^\circ$) ($p < 0.05$). Thus, EFMs with substantial variations in hydrophobicity were produced through surface treatment (e.g., superhydrophobic: Fluorinated EFMs, hydrophobic: PCL EFMs, and superhydrophilic: PCL/PEGPCL and PCL-AAc EFMs).

Table 1. Comparison of variables of EFMs

	Water contact angle (°)	Average fiber width ω (μm)	Mean pore size (μm)	Porosity (%)
PCL (300 μm)	135.2 ± 5.4	1.726 ± 0.102	1.517 ± 0.203	82 ± 2
PCL (800 μm)	146.5 ± 6.1	1.591 ± 0.082	1.689 ± 0.211	75 ± 4
PCL (1100 μm)	149.6 ± 2.0	1.651 ± 0.039	1.572 ± 0.166	82 ± 1
Fluorinated PCL	157.0 ± 2.4	1.602 ± 0.093	1.678 ± 0.074	75 ± 3
PCL/PEGPCL	~ 0	1.512 ± 0.141	1.557 ± 0.229	83 ± 2
PCL-AAc	~ 0	1.556 ± 0.170	1.435 ± 0.492	80 ± 3

3.3 Conclusions

Diacryl-PEGPCL and diacryl-PEGPLA block copolymers were successfully synthesized with average yields of 83.6% and 85.4%, respectively. PCL (300 μm , 800 μm , and 1100 μm thick), PCL/PEGPCL core/shell, fluorinated PCL, and acrylic acid (AAc) treated PCL (PCL-AAc) EFMs were fabricated and characterized. All EFMs displayed similar average fiber width, mean pore size and porosity, whereas substantial variations in hydrophobicity were found (superhydrophobic: Fluorinated EFMs, hydrophobic: PCL EFMs, and superhydrophilic: PCL/PEGPCL and PCL-AAc EFMs).

Chapter 4: Controlled Release Studies of Hydrogel-Electrospun Fiber Mat Composite Materials²

Because of their hydrophilicity, biocompatibility, and tunable mechanical properties, hydrogels have been used extensively for controlled release of macromolecules, particularly hydrophilic proteins [57, 69, 71, 80, 178, 292-294]. However, despite promising advances in drug residence time and sustained delivery rates, hydrogels have not attained broad clinical application. Two limitations to their use in the clinic are unpredictable initial rapid release (i.e., burst release), especially of low molecular weight drugs, and uncontrolled release rates. Burst release results from a number of factors [281, 295]. For example, low molecular weight drugs are often substantially smaller than hydrogel mesh sizes, resulting in rapid diffusional loss of entrained compound. When these hydrogels are placed in a new aqueous environment with low drug concentration, drug rapidly desorbs from the surface driven by the high solution concentration gradient. In hydrogels that specifically resist protein adhesion, such as PEG, one suggested explanation for burst release is the presence of drugs at the hydrogel surface as a result of surface entrapment during the photo-polymerization process [296]. This has especially been noted when high loading of small (e.g., low molecular weight) drugs is used. Also, for block co-polymer-based hydrogels, the partitioning of proteins between the hydrophobic domain and hydrophilic domain plays

² Portions of this material have been submitted to Journal of Controlled Release and Journal of Biomedical Materials Research Part A.

an important role in initial burst release [296, 297]. During the gelation process, part of the aqueous phase will be expelled as the system volume contracts in the solution-gel transition region. Drugs dissolved in the expelled solution will be forced to migrate to the surface or near-surface regions and contribute to the burst effect. On the other hand, drugs associated with or dissolved in the hydrophobic domain will not be expelled. Although burst release can be advantageous in limited situations, such as wound treatment [298] and encapsulated food flavors, burst release is generally undesirable because sharp increases in drug concentration can cause local or systemic toxicity and unnecessary waste of expensive therapeutic compounds [299, 300].

The second major limitation is inability to control hydrogel drug release rates. Many applications require drug release “on-demand”, and several smart polymer systems have been developed for this purpose [80, 86, 87]. In other applications, linear drug release may be desired. For example, release of chemotherapeutics for cancer therapy [301], anti-inflammatory compounds to treat degenerative disease [61], and therapeutics for ophthalmological use [302] may demand linear drug release. Unfortunately, non-linear release from hydrogel systems is not surprising. A material that follows standard, concentration gradient-driven Fickian diffusion will exhibit greater release rates initially when the concentration gradient is high and declining release rates as the concentration reaches equilibrium. Release rates can be modified using degradable materials, which exhibit mesh sizes that increase with time thereby producing a time-dependent diffusion coefficient; however, these typically do not approach linear release [292, 303]. Non-linear release is often undesirable, especially for drug delivery, because many

therapeutics require constant dosing over time [281, 302]. Dosing excursions can result in toxicity or inadequate treatment in patients.

Modification of hydrogel release rates has been the focus of vigorous research in recent years and has followed several approaches. For example, to reduce burst release, drugs can be removed from the hydrogel surface by extraction before use [57, 304]. This method requires additional processing steps and results in loss of some therapeutic compound. Release can also be delayed or slowed by direct modification of the hydrogel surface [305, 306] or addition of semi-impermeable coatings [307-309]. These experiments have demonstrated that the area of exposed surface and surface characteristics have a significant impact on hydrogel swelling, water penetration, and drug diffusion [307-309]. Alternatively, non-uniform drug loading can be used to modify release rates [304, 310]. For example, if drug is primarily concentrated in the hydrogel interior, release can be delayed. One of the most popular methods to diminish burst release and produce uniform release rates has been to alter the hydrogel matrix itself. Polymer morphology, composition [311, 312], and physical and chemical properties [123, 302, 313] have all been explored as variables in release kinetics; and in particular, hydrophilic/hydrophobic interactions have been shown to dramatically alter release rates [123, 302, 313]. This observation has been exploited to create composite drug delivery vehicles that combine hydrogels with other release systems (e.g., micro- and nanoparticles) to reduce initial burst release and extend release duration [177, 178, 314].

In this chapter, we explore the possibility of combining bulk hydrogels with EFMs for controlled molecule delivery. EFMs, thin (100-1000 μm) porous mats of randomly oriented electrospun nanofibers ($\sim 100\text{ nm}$ -3 μm diameter), have been widely

explored as drug carriers in tissue engineering [79, 225, 282, 315, 316]. They have previously been combined with hydrogel beads (e.g., gelatin-heparin, a hyaluronic acid derivative) to yield composite delivery systems [316]. Our approach complements these efforts by examining the macroscopic coupling of large EFMs with bulk hydrogels.

In the first section of this chapter, we present a hydrogel-EFM composite release system that exploits surface properties and hydrophilic/hydrophobic interactions to dramatically improve release kinetics. This system, which was inspired by previously reported hydrogel-microparticle composites [178], consists of PEGPCL hydrogels coupled with PCL EFMs through ultra-violet (UV) photo-polymerization. PEGPCL hydrogels were selected for their extended release duration, biocompatibility, and biodegradability. Additionally, because of their formation through photo-crosslinking, they can be formed into a variety of shapes and sizes [83, 91, 317, 318] and easily coupled to EFMs. PCL EFMs were selected for their chemical similarity to the hydrogel materials employed (PCL vs. PEGPCL) and because of their hydrophobic properties. EFMs can be cut to virtually any macroscopic shape, thus restricting the exposed hydrogel surface. When composite materials are constructed in a sandwich configuration (EFM+Gel+EFM), the external EFMs provide an additional diffusion barrier that significantly alters release kinetics. Also, composite materials (EFM+Gel+EFM) demonstrated more linear release with increased duration (70 vs. 40 days) and a significant reduction in burst release (7% vs. 20%) compared to unmodified hydrogels. The improved release profiles observed for hydrogel-EFM composites can most likely be attributed to either the hydrophobicity of the EFMs employed or the increased diffusional path length imparted by their presence.

To evaluate these hypotheses, we next examined the effect of EFM thickness and hydrophobicity on hydrogel swelling and therapeutic release. (Hydrogel swelling is closely linked to release behaviors [307, 308, 319].) EFMs with different thickness (i.e., 300, 800 and 1100 μm PCL EFMs) and hydrophobicity (i.e., PCL, fluorinated PCL, PCL/PEGPCL core/shell, and acrylic acid (AAc) treated PCL EFMs) were synthesized as described in Chapter 3, and combined with PEG-poly(lactic acid) (PEGPLA) hydrogels. The release of model protein bovine serum albumin (BSA) from these materials was examined. We found that increasing either EFM thickness or hydrophobicity resulted in longer and more linear release profiles with reduced initial burst release, demonstrating the remarkable applicability of this new system.

4.1 Materials and methods

4.1.1 PEGPCL hydrogel- PCL EFM release kinetics

4.1.1.1 Construction of PEGPCL hydrogel-PCL EFM composite materials with different configurations

PEGPCL hydrogels were created by UV polymerization, following the method of Hubbell et al. [82]. A 22% w/v solution of macromer in Dulbecco's phosphate buffered saline (D-PBS, Sigma) was used. For hydrophilic protein release tests, 5% wt. bovine serum albumin (BSA, Invitrogen) was added to the macromer solution before UV exposure. A mixture of Irgacure 2959 (0.1% wt, Ciba, Tarrytown, NY) and 1-vinyl-2-pyrrolidone (0.5% wt, Sigma) was used as an initiator. Samples were prepared in 8 mm wells (8 well SecureSealTM hybridization chamber (Grace Bio-Labs, SA8R-2.5)) (50 μL of precursor solution/well) and exposed to UV illumination (Blak-Ray, B-100-AP, UVP) for 7 min to form gels.

To identify the effect of EFM position in the hydrogel phase on the protein release profile, PEGPCL hydrogel-PCL EFM composite delivery systems were constructed in two different configurations (Figure 18). In composite A (Gel+EFM+Gel) (Figure 18A), PEGPCL hydrogels were formed in 8 mm wells (8 well SecureSeal™ hybridization chamber (Grace Bio-Labs, SA8R-2.5)) (25 μ L precursor/ well). After 4 min of UV illumination, an 8 mm-diameter PCL EFM was placed on the top surface of the hydrogel (one EFM/well), and an additional 25 μ L of PEGPCL precursor were placed on top of each EFM. Samples were exposed to UV illumination for 5 additional minutes to complete hydrogel formation. To construct composite B (EFM+Gel+EFM) (Figure 18B), 8 mm diameter PCL electrospun fiber mats were placed on the bottom of 8 mm wells (one EFM/well) and PEGPCL macromer solution (50 μ L precursor/well) was added. After 6.5 min UV illumination, a second PCL EFM was placed on the top surface of each hydrogel. The resulting composite system B was exposed to UV illumination for 1 additional min. All samples were formed in triplicate.

Gelation times were selected based on initial trials with PEGPCL hydrogel, composite A, and composite B materials following the method of Sawhney et al. [4]. Briefly, we continuously scratched the surface of the macromer solution with a sharp needle until an evident scratch mark was seen. For composite fabrication, we first measured the required UV exposure time for macromer solution gelation. Then, we determined the minimum amount of UV exposure for the same volume of macromer solution that permitted an EFM to be supported in the middle (composite A) or on the top (composite B) of the hydrogel without loss of cohesion after submersion in PBS buffer. It is also possible that differences in gelation times could result in the observed release

kinetics; however, we have seen that mat thickness and hydrophobicity alter release kinetics in samples with identical gelation conditions, which suggests that kinetics are strongly influenced by EFM properties. In the following Section 4.1.2, this phenomenon was investigated in more detail.

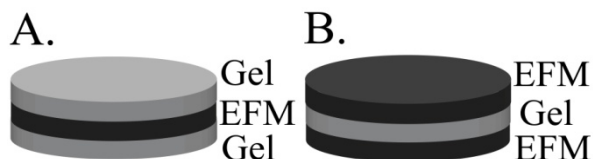


Figure 18. Scheme of PEGPCL hydrogel-PCL EFM composite materials (Gray: PEGPCL hydrogel, black: PCL EFM).

4.1.1.2 Release kinetics of PEGPCL hydrogel-PCL EFM composite materials

To evaluate release properties of hydrogel-EFM composites, we examined release of a model protein, BSA, over several weeks. PEGPCL hydrogel-EFM composite delivery systems containing BSA model protein were placed in pH 7.4 Dulbecco's-PBS (containing 0.1% methylparaben (Sigma) as a preservative) at 37 °C. Aliquots of supernatant were withdrawn every other day for over two months and replaced with an equal amount of fresh PBS. To ensure an approximately infinite release environment, at least a 20 fold excess of D-PBS to sample volume was used, and withdrawn samples comprised no more than 7% of the total solution volume [303]. Actual release duration was based on gel integrity; data collection was halted after intact hydrogels or composites could no longer be identified in solution. Protein release has a strong correlation with hydrogel degradation in PEGPCL polymers [320], and therefore likely closely correlates with degradation rate.

BSA release was characterized using the Bradford total protein assay and reported as fractional release vs. time. M_m , the mass of BSA released at time point t_n , was calculated from:

$$M_m = C_m \times 1 + 0.04 \times (C_{t1} + \dots + C_{t(n-1)}) \quad \text{Eq. (4.1)}$$

where the later factor accounts for BSA removed during sample collection. BSA fractional release was expressed as $\frac{M_t}{M_{inf}}$, where M_t and C_t are the mass and the concentration of released BSA at time point t , and M_{inf} is the total mass of BSA eluted at equilibrium.

4.1.1.3 Study of aggregation of BSA released from composite materials through Native PAGE

The aggregation status of BSA eluted from composite B (EFM+Gel+EFM) was measured using Native PAGE (polyacrylamide gel electrophoresis, Ready-Gel Tri-HCl Gel (12% precast polyacrylamide gel), Mini-PROTEAN system, Bio-Rad Laboratories).

4.1.1.4 Composite B (EFM+Gel+EFM) *in vitro* Biocompatibility

Gross biocompatibility of hydrogel-EFM composite materials was evaluated using PC12 cells (American Type Culture Collection (ATCC), CRL-1721, Manassas, VA), a commonly studied neuronal precursor cell line derived from a pheochromocytoma of the rat adrenal medulla [321]. Cells were cultured at 1×10^4 cells/cm² on collagen-coated (BD Bioscience, 5 µg/cm²) 24-well tissue-culture plates and incubated overnight in 1.5 mL standard culture medium (Ham's F12K medium with 2mM L-glutamine, 1.5 g/L sodium bicarbonate, 2.5% fetal bovine serum (FBS), 15% horse serum (all from Sigma-Aldrich), and 1% penicillin-streptomycin (Gibco, Carlsbad, CA)). Composite B

scaffolds (EFM+Gel+EFM) containing 5 wt% BSA were immersed in PBS buffer for one hour to remove surface adsorbed drug and cell culture medium for another hour to enhance biocompatibility. Composites were then placed on the upper surface of membrane Transwell™ inserts (Corning, NY) to eliminate direct interactions between the composite scaffolds and cells, as shown in Figure 19. Cells were cultured in the presence of composite B for 7 and 14 days. The MTT assay (Sigma Aldrich), which measures mitochondrial enzyme ability to convert 3-(4, 5-di-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan crystals, was used to assess cell viability. Samples were compared to sham having cells cultured with no-BSA composite materials (N=3), and negative controls having cells cultured without composite materials (N=3). Each sample was evaluated using a Versamax UV-visible microplate reader in triplicate. One way ANOVA was used for statistical analysis.

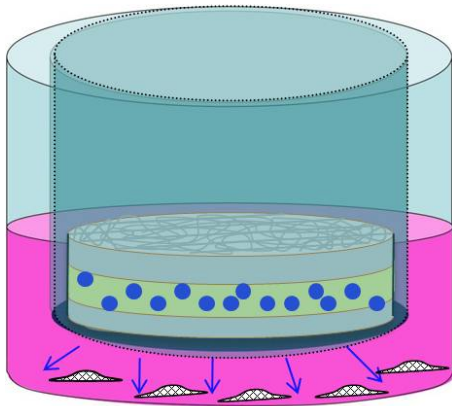


Figure 19. Scheme of composite B *in vitro* bioactivity test (blue circles: BSA, not scale to actual size).

4.1.1.5 Study of bioactivity of nerve growth factor (NGF) released from composite B materials in a PC12 cell culture model

PC12 cells exhibit a spread, neuronal morphology with neurite extension in the presence of NGF, but revert to a rounded morphology with few extensions in the absence of NGF. Thus, neurite extension can be used as a measure of the quality of released drug (i.e., NGF). PC12 cells were cultured at 1×10^4 cells/cm² in collagen-coated 24-well tissue-culture plates overnight. BSA and NGF were added to the hydrogel phase of composite B materials (5 wt% BSA and 100 ng NGF/ μ L in 50 μ L hydrogel). After immersion in PBS buffer and cell culture medium for one hour each, composite B materials were placed on the upper surface of Transwell™ membrane inserts in the presence of PC12 cells. Medium was exchanged every 2~3 days. Neurite extension was evaluated using phase contrast microscopy (10 and 20 $\times$) at 7 and 14 days. Five pictures were taken randomly from each sample well (N=3). Neurite lengths (measured from the soma to the tip of each neurite branch) and number densities were assessed by Image J software (NIH free software). NGF- negative controls (cell culture medium without NGF) (N=3) and NGF+ positive controls (NGF directly added into cell culture medium, 50ng/mL) (N=3) were also studied for comparison. Because neurite extension is known to have a non-Gaussian statistical profile, the Kruskal-Wallis test (for non-parametric data) was used for statistical analysis.

4.1.2 Swelling and release behaviors of PEGPLA hydrogel-EFMs composite materials

To form composites, PCL, PCL/PEGPCL core/shell, fluorinated PCL, and acrylic acid (AAc) treated PCL (PCL-AAc) EFMs were cut into circular discs with a diameter of 8 mm using dermal punches (Acuderm). PEGPLA hydrogel-EFM composite materials

were constructed as described in Section 4.1.1.1 (Figure 18B). PEGPLA hydrogels were used in this study instead of comparative molecular weight PEGPCL because of their faster degradation rate [322, 323]. To evaluate composite protein release, BSA was used as a model protein, and incorporated into PEGPLA hydrogels and the composite PEGPLA hydrogel layers. Since BSA is a hydrophilic protein, its concentration distribution in hydrogels was assumed to be uniform.

4.1.2.1 Swelling studies of PEGPLA hydrogel-PCL EFMs of different thicknesses and hydrophobicity

PEGPLA hydrogels and hydrogel-EFM composite materials ($N = 3$) were placed in 1.5mL microcentrifuge tubes (one scaffold per tube, immersed in 1 mL double deionized H_2O), and incubated at $37^\circ C$. Then, the thickness (δ_s) and diameter (D_s) of swollen scaffolds were measured using a caliper every other day. No evident change of the thickness and diameter of swollen scaffolds was observed after one day incubation. Subsequently, samples were dried in a vacuum oven for 2 hours. The thickness (δ_d) and diameter (D_d) of dried scaffolds were also evaluated. To compare swelling behavior in different composite materials, the ratios of thickness, total area, and volume of swollen and dried scaffolds were calculated using the following equations [324]:

$$\text{Thickness ratio} = \delta_s / \delta_d \quad \text{Eq. (4.2)}$$

$$\text{Area ratio} = A_s / A_d = \frac{2D_s\delta_s + D_s^2}{2D_d\delta_d + D_d^2} \quad \text{Eq. (4.3)}$$

$$\text{Volumetric swelling ratio} = V_s / V_d \quad \text{Eq. (4.4)}$$

4.1.2.2 Release studies of PEGPLA hydrogel-PCL EFM composites of different thicknesses and hydrophobicity

Composites with EFMs of different thicknesses and hydrophobicities were synthesized as described in Section 3.1.2. Similar to the procedures described in Section 4.1.1.2, BSA-containing PEGPLA hydrogels and composite materials (N = 3) were placed in 1.5 mL low-retention microcentrifuge tubes (containing 1 mL pH 7.4 D-PBS with 0.1 % methylparaben added as a preservative). 40 μ L aliquots of supernatant were withdrawn and replaced with 40 μ L fresh D-PBS regularly for over two months. The Bradford total protein assay was applied to quantify the concentration of eluted BSA. BSA cumulative release was then calculated using Equation (4.1). The effects of EFM hydrophobicity and thickness were analyzed using a design of experiment factorial fit with the least squares method (JMP statistical software 8.0) to identify individual contributions of these two factors.

4.2 Results and discussion

4.2.1 Effect of different EFM locations on PEGPCL hydrogel-PCL EFM composite material release kinetics

Drug released from composite materials with different configurations was examined using BSA as a model protein (Figure 20A, Table 2). PEGPCL hydrogel control and composite A (Gel+EFM+Gel) demonstrated typical hydrogel release curves with a modest burst of ~20% and diminishing release over ~40 days. There was no detectable difference between PEGPCL hydrogel and composite A release profiles, which implies that EFM placement in the center of the hydrogel has no significant effect.

Composite B (EFM+Gel+EFM); however, exhibited substantially altered release kinetics with a more linear release curve and extended release duration (i.e., over 2 months).

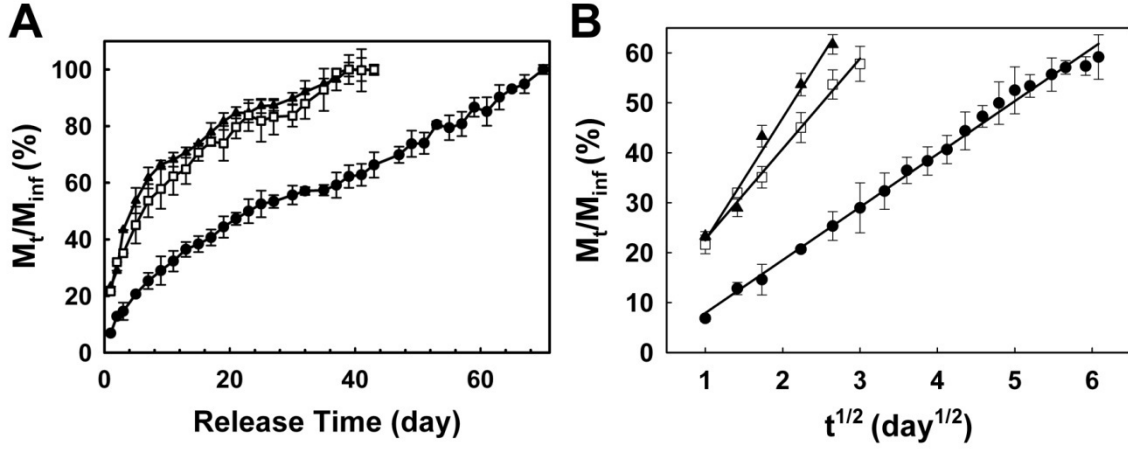


Figure 20. (A) *In vitro* release profiles of BSA and (B) first 60% fractional release of BSA from (▲) PEGPCL hydrogels, (□) composite A (Gel+EFM+Gel), and (●) composite B (EFM+Gel+EFM); R^2 values for (B) (▲) PEGPCL hydrogels (0.974), (□) composite A (0.977), and (●) composite B (0.990).

The release mechanism of these systems is complicated and influenced by polymer degradation, hydro-phobicity/phility, hydrogel swelling, and solute diffusion. As a first approximation, the power law model (Equation (4.5)) developed by Ritger and Peppas [325] was applied to analyze the first 60% of fractional drug release:

$$\frac{M_t}{M_{inf}} = kt^n \quad \text{Eq. (4.5)}$$

where M_t is the mass of drug released at time t , M_{inf} is the total mass of drug released at equilibrium, k is a constant depending on both D , the drug diffusivity, and l , the characteristic length of the polymer, and n is the diffusional exponent, which is related to the diffusion coefficient and the specific transport mechanism (0 for no release, 0.5 for Fickian diffusion, $0.5 < n < 1$ for anomalous diffusion, and 1 for zero-order or case II release from thin swellable polymer slabs with one-dimensional diffusion under perfect

sink conditions [325]). Constant k and diffusion exponent n values can be obtained by fitting M_t/M_{inf} versus t data with the power law model.

To obtain the effective diffusion coefficients (D_{eff}), a simplified version of Fick's Law for diffusion from a plane surface for short times ($\frac{M_t}{M_{inf}} < 60\%$) was applied [303]:

$$\frac{M_t}{M_{inf}} = 4 \left[\frac{D_{eff} t}{\pi \delta^2} \right]^{1/2} \quad \text{Eq. (4.6)}$$

where δ is the thickness of PEGPCL hydrogel or composite material.

Replacing the right hand side with $k' \sqrt{t}$:

$$\frac{M_t}{M_{inf}} = k' \sqrt{t}, \quad k' = 4 \left[\frac{D_{eff}}{\pi \delta^2} \right]^{1/2} \quad \text{Eq. (4.7)}$$

The plot of M_t/M_{inf} versus $t^{1/2}$ is shown in Figure 20B, and the slope of the fitted curve approximately represents the value of k' .

D_{eff} can be obtained via the Equation (4.8):

$$D_{eff} = \frac{\pi (k' \delta)^2}{16} \quad \text{Eq. (4.8)}$$

Release kinetics of BSA released from composite A and B were compared in Table 2.

As shown in Figure 20 and Table 2, release duration and k constant values for PEGPCL hydrogels and composite A (Gel+EFM+Gel) were nearly identical. However, the diffusional exponent n decreased for composite A compared to PEGPCL hydrogels. This suggests that the incorporation of a PCL EFM into the center of the hydrogel slab slightly slowed the first 60% of BSA release, most likely in the vicinity of the EFM. Composite B showed dramatically increased release duration (70 vs. 40 days) with a far more linear release profile and substantially reduced burst release. The mechanism

responsible for this relatively steady and extended release remains unclear, however, may result from the structure and properties of the EFMs employed. Composite B exhibited increased values for the diffusional exponent n and significantly reduced values for constant k when compared with PEGPCL hydrogels and composite A. The increase in diffusional exponent, n (0.581), represents a slight deviation from Fickian behavior ($n=0.5$) and is not drastically altered from values of PEGPCL hydrogel (0.527) and composite A (0.443). Thus, it is the significant decrease in constant k that suggests a mechanism for altered release from these composites.

Table 2. BSA release from hydrogels and hydrogel-EFM composites

	PEGPCL Hydrogel	Composite A (Gel+EFM+Gel)	Composite B (EFM+Gel+EFM)
Release Time(days)	40	40	70
Burst Effect	23.3%	21.6%	6.8%
Constant k (day ^{-1/2})	0.225	0.222	0.079
Diffusion Exponent (n)	0.527	0.443	0.581
Diffusion Coefficient ($D_{\text{eff}}/(10^{-3}\text{mm}^2/\text{day})$)	11.03	9.46	3.47

Constant k is dependent on drug diffusivity and diffusion path length, both of which could be influenced by the presence of EFMs. EFMs used were ~0.8 mm thick, whereas hydrogel slabs were ~1 mm thick, thus the addition of EFMs nearly tripled the half-length thickness of the composite (1.3 mm vs. 0.5 mm). The diffusional path length is also dependent on tortuosity, which would differ for hydrogels (i.e., average mesh size ~ 10 nm) and EFMs (i.e., average pore size ~ 100 nm), so this is only a rough

approximation of the potential effect. It should also be noted that because model drug BSA was only incorporated in the hydrogel phase, the initial drug loading distribution of composite B was significantly different from the uniform drug distribution in PEGPCL hydrogel and near-uniform loading of composite A systems (excepting drug excluded from the central EFM). Thus for composite B, drug had to navigate through a non-drug-containing EFM before reaching solution, whereas both composite A and PEGPCL hydrogels had drug-containing phases directly exposed to solution. In addition to increasing the diffusional path length, EFMs are hydrophobic and thus decreased D_{eff} for the hydrophilic BSA model protein ($3.47 \times 10^{-3} \text{ mm}^2/\text{day}$ vs. $11.03 \times 10^{-3} \text{ mm}^2/\text{day}$ for PEGPCL hydrogel) through hydrophilic-hydrophobic repulsion. Hydrophobic EFMs probably also reduced hydrogel water uptake and therefore hydrogel degradation rate, resulting in delayed release. However, as the EFM is also degradable, these effects are time dependent and degradation/release would be hindered more near the beginning of the cycle when the EFM is fully intact than at later stages. The combination of these factors results in an extended, more linear release curve compared with PEGPCL hydrogels and composite A. These same factors are probably also responsible for the reduced burst effect (7% vs. 20%), as hydrophilic drug must navigate an increased, hydrophobic diffusional path length.

4.2.2 Bioactivity of protein eluted from PEGPCL hydrogel-PCL EFM composite B

In addition to release studies, we also evaluated the potential of hydrogel-EFM composites for *in vitro* drug release. Aggregation status of BSA eluted from composite B (EFM+Gel+EFM) was evaluated using Native PAGE (polyacrylamide gel

electrophoresis) (Figure 21, Table 3). Aggregated protein often displays decreased bioactivity, which is undesirable in drug delivery applications. In the first 5 days, sample monomer, dimer, and trimer protein bands displayed little difference from the unencapsulated control, indicating no detectable aggregation of eluted BSA. This may result from the small cumulative release during that period. After 6 weeks, some BSA aggregation was observed. At Day 70, about 40% of eluted BSA was aggregated vs. 31% in the control sample. This represents a modest increase (~9%) in protein aggregation upon release from the hydrogel-EFM composite.

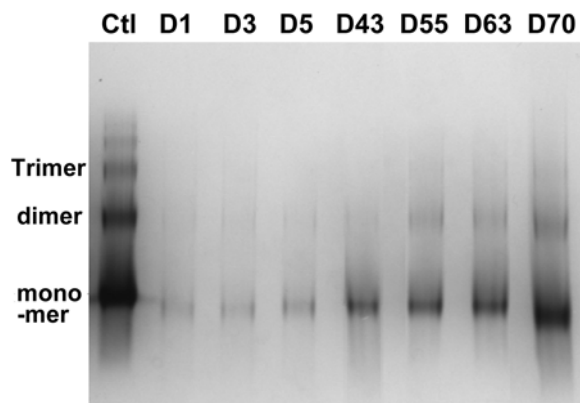


Figure 21. Native PAGE of BSA released from composite B (EFM+Gel+EFM). DX=Day after composite formation

Table 3. BSA aggregation status

Release time (day)	0 (Ctl)	43	55	63	70
Aggregation status (%)	30.6	34.3	36.9	38.7	39.5

Next, we evaluated the potential of composite B to release bioactive drugs in cell culture. Gross cytotoxicity of composite B (EFM+Gel+EFM) was examined using the MTT assay and model PC12 neuronal cells. No significant differences in cell viability

was observed for sham (no-BSA, C), BSA-eluting composites (CB), and control cells after two weeks ($p=0.83$, $N=3$) (Figure 22).

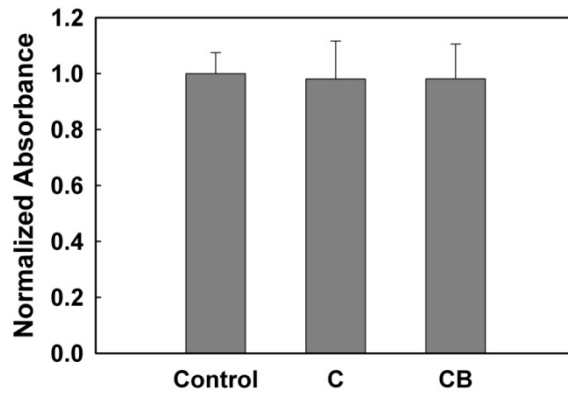


Figure 22. Normalized absorbance from an MTT cell viability test.

We then examined nerve growth factor (NGF) release to PC12 cells, which are known to reversibly stop proliferating and differentiate into a neural phenotype in response to NGF exposure. Bioactivity was assessed by examining PC12 cell neurite extension using phase contrast microscopy. Samples consisted of a positive control receiving 50 ng/mL NGF, composite B releasing NGF, a negative control receiving 0 ng/mL NGF, and a BSA-releasing sample (Sham). Data were collected at 7 and 14 days (Figure 23).

Both the positive control and NGF-releasing composite (Figure 23A and B, respectively) contained cells exhibiting a neuronal phenotype with accompanying neurite extension at day 14, whereas negative control and sham (Figure 23C and D, respectively) samples maintained an undifferentiated, rounded morphology, without measurable neurite extension. Additionally, in the absence of NGF, PC12 cells proliferated, producing large cell aggregates (Figure 23C and D).

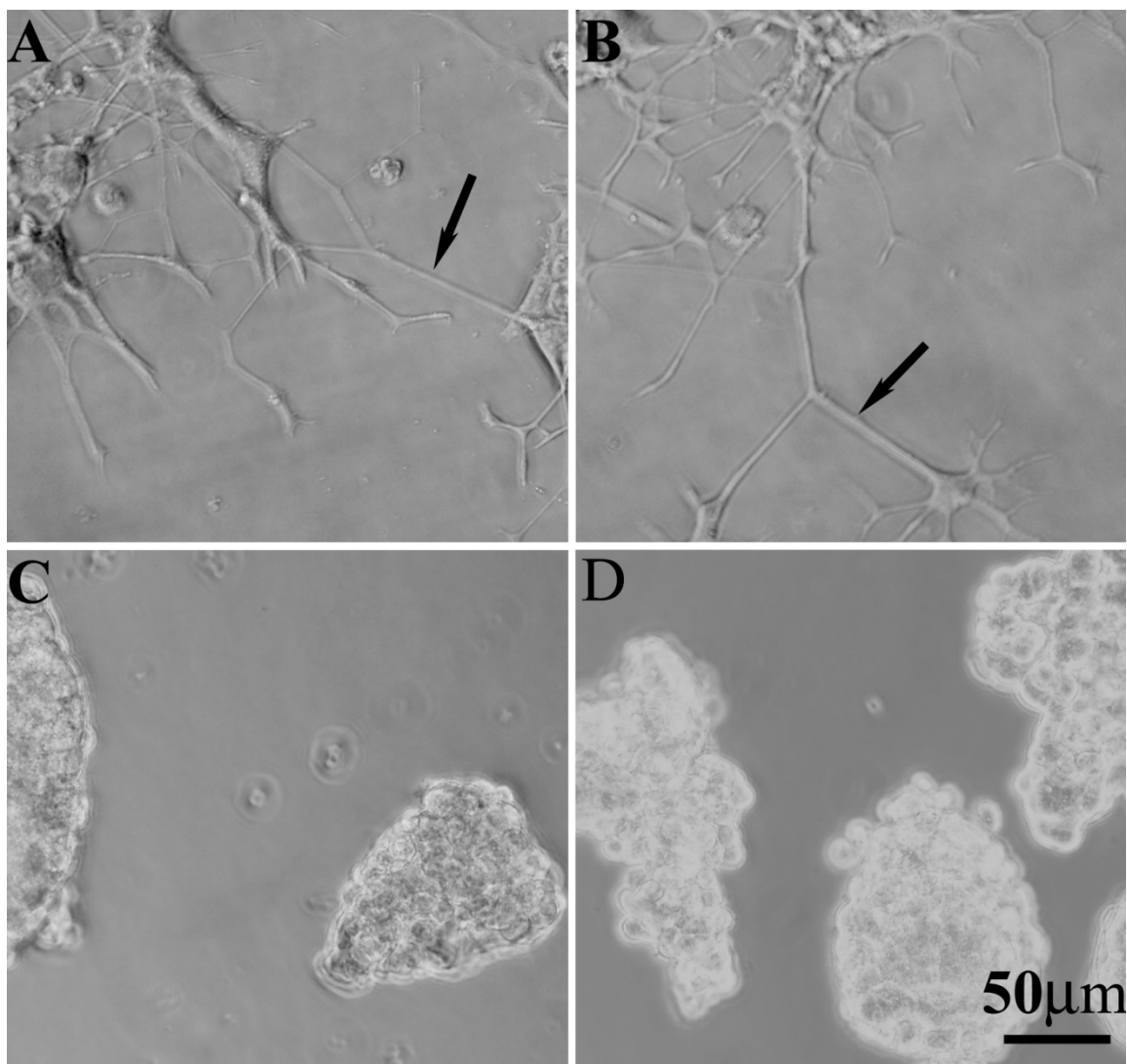


Figure 23. (A-D) Representative phase contrast micrographs of PC12 cells after 14 days in culture (A) positive control receiving 50 ng/ mL NGF, (B) NGF-releasing composite B sample, (C) negative control receiving 0 ng/ mL NGF, (D) BSA-releasing sample (sham). Arrows indicate extended neurites.

Neurite length distributions for the NGF+ control and composite B (N=3) at days 7 and 14 were also compared (Figure 24). Neurite length distributions at day 7 are statistically different ($p<0.05$) for both samples from those at day 14. Since the differentiation of PC12 cells is dose-dependent [272, 326], increases in average neurite length from day 7 to 14 indicate sustained release of bioactive NGF from composite B materials.

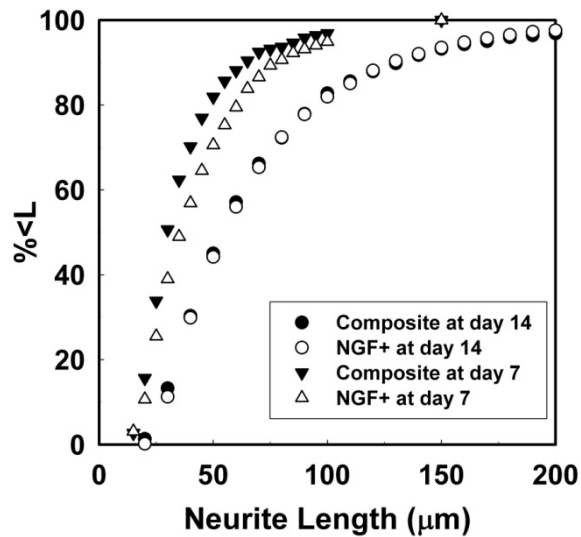


Figure 24. Percent of neurites possessing length $< L$ at day 7 and 14.

PC12 cells responded to NGF-releasing composite B materials by exhibiting more, shorter neurites ($p < 0.05$) than in the NGF+ control after 7 days culture (Figure 24, 25A). Interestingly, there was no statistical distinction ($p > 0.05$) between neurite extension distributions of composites and the NGF+ control at day 14 (Figure 25B). Thus, the amount of released NGF was sufficient to stimulate PC12 cell neurite extension for at least 14 days in a manner similar to a positive control.

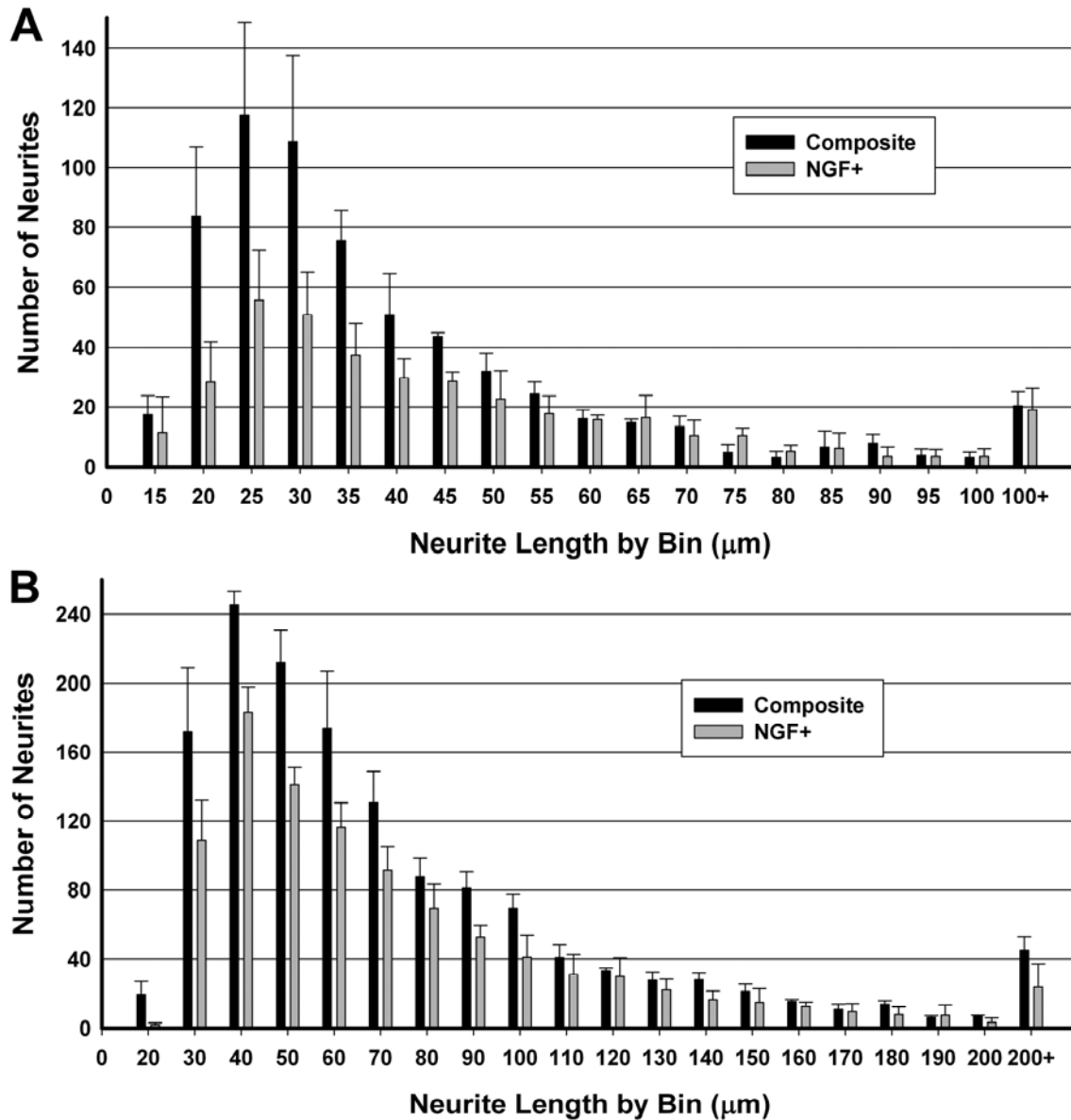


Figure 25. Histograms of neurite extension status at day 7 (A) and day 14 (B).

4.2.3 Swelling behavior of PEGPLA hydrogel-EFM composites

To obtain better understanding of the primary causes for the more linear and extended release profiles of composite materials in the composite B (EFM+Gel+EFM) configuration, we applied modified PLA EFMs with different thicknesses and hydrophobicity to construct composite B materials, and studied the swelling and release

behaviors of these samples. Since initial hydrogel drug release is closely related to swelling; thickness, total area, and volumetric swelling ratios of swollen and dried PEGPLA hydrogels and PEGPLA hydrogel-EFM composite materials were investigated (Figures 26-28). After one day incubation in water, thickness ratios (δ_s/δ_d) generally increased with increasing hydrophobicity (Figure 26), although significant differences were only evidenced at the extremes (e.g., for superhydrophobic fluorinated EFM-hydrogel composites and superhydrophilic PEGPLA hydrogels). Total area ratio (A_s/A_d) (Figure 27) followed a similar trend with increases correlated to increasing EFM hydrophobicity; however, unmodified PEGPLA hydrogels displayed significantly higher area ratios than any other material investigated, nearly double that of EFM-hydrogel composites. This suggests that the presence of EFMs in the composite significantly constrained swelling in the radial direction (r). In addition, it is likely that increased swelling in the z direction observed with increasing hydrophobicity (Figure 26) can be attributed to reduced interpenetration of the hydrophilic hydrogel into the hydrophobic EFM network. It is also possible that these increases result from direct swelling of the EFMs (unlikely for hydrophobic EFMs) or from enhanced relaxation in the axial direction (z) given the swelling constraint in the radial direction (r). However this latter scenario is not supported by Figure 27 in which hydrophobic EFM composites display the least radial constraint but the greatest increase in axial swelling.

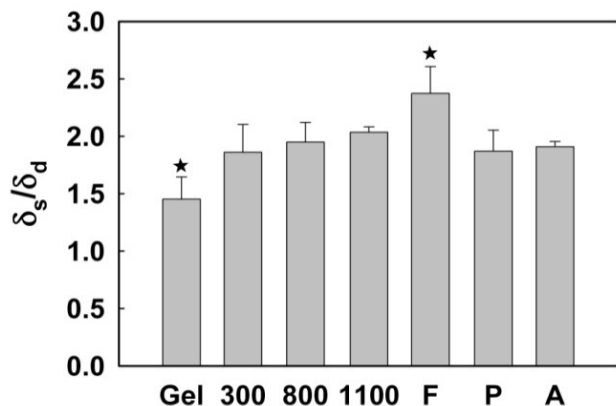


Figure 26. Thickness ratios of swollen and dried PEGPLA hydrogel and composite materials. (Gel: PEGPLA hydrogels, 300, 800, 1100: composite materials with 300, 800 or 1100 μm PCL EFMs, F, P, A: composite materials with fluorinated PCL, PCL/PEGPCL core/shell, or PCL-AAc EFMs) (Asterisks indicate significant differences).

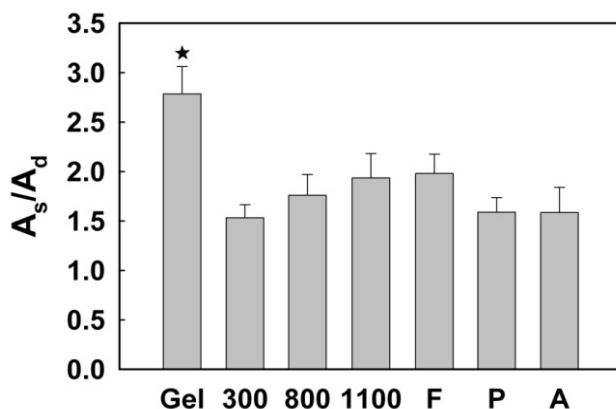


Figure 27. Total area ratios of swollen and dried PEGPLA hydrogel and composite materials. (Gel: PEGPLA hydrogels, 300, 800, 1100: composite materials with 300, 800 or 1100 μm PCL EFMs, F, P, A: composite materials with fluorinated PCL, PCL/PEGPCL core/shell, or PCL-AAc EFMs) (Asterisk indicates significant difference.)

Consistent with this hypothesis, PEGPLA hydrogels evidenced higher volumetric swelling ratios than all composite materials investigated (Figure 28). Composite material swelling ratios were closely related to EFM hydrophobicity, with composites comprised of thinner and more hydrophilic EFMs displaying reduced swelling. This supports the theory that more hydrophilic EFMs may encourage the formation of a better interface

with hydrophilic hydrogels, which in turn constrains hydrogel swelling in the radial direction, r (Figure 27), and to a lesser extent z (Figure 26).

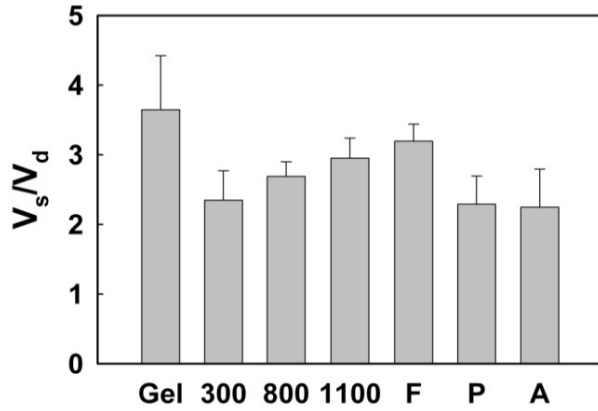


Figure 28. Volumetric swelling ratios of PEGPLA hydrogel and composite materials. (Gel: PEGPLA hydrogels, 300, 800, 1100: composite materials with 300, 800 or 1100 μm PCL EFMs, F, P, A: composite materials with fluorinated PCL, PCL/PEGPCL core/shell, or PCL-AAc EFMs.)

4.2.4 Release behavior of PEGPLA hydrogel-EFM composites

The fractional release of the first 60% of model protein BSA from PEGPLA hydrogels and composite materials was evaluated as a function of PCL EFM thickness (Figures 29A and B). The addition of EFMs to PEGPLA hydrogels extended the release period for the first 60% BSA from 10 days (PEGPLA hydrogels) to 20 (300 μm PCL EFMs), 24 (800 μm PCL EFMs), and 35 days (1100 μm PCL EFMs) (Figure 29A). Thus, the 60% BSA release period increased with increasing PCL EFM thickness. Similarly, the BSA release rate, which is proportional to the slope of the release curve (M_t/M_{inf} vs t , Figure 29A), decreased with increasing PCL EFM thickness.

Fractional release of the first 60% BSA was also evaluated as a function of EFM hydrophobicity (Figures 30A and B) for PEGPLA hydrogels and composite materials with PCL-AAc, PCL/PEGPCL core/shell, PCL and fluorinated PCL EFMs (1100 μm).

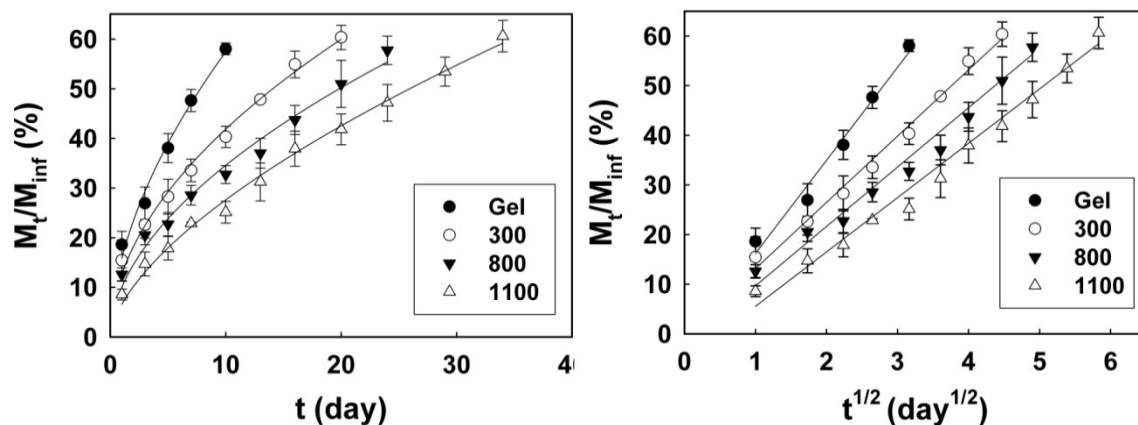


Figure 29. First 60% fractional release of BSA from hydrogel and composite materials comprised of EFMs with different thicknesses. (● PEGPLA hydrogel; ○ composite materials with 300 μm ; ▼ 800 μm ; and △ 1100 μm PCL EFMs. Lines represent fit to the power law model (Eq. 4.5) (A) and Fick's law (Eq. 4.6) (B), $R^2 > 0.95$ for all fitting curves.)

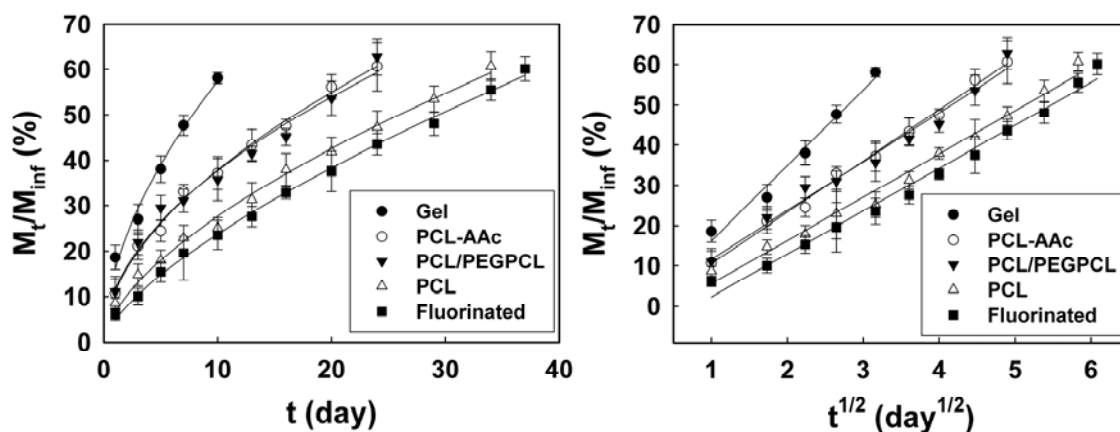


Figure 30. First 60% fractional release of BSA from hydrogel and composite materials comprised of EFMs with different hydrophobicity. (● PEGPLA hydrogel; ○ composite materials with PCL-AAc; ▼ PCL/PEGPCL core/shell; △ PCL; and ■ fluorinated PCL EFMs. Lines represent fit to the power law model (Eq. 4.5) (A) and Fick's law (Eq. 4.6) (B), $R^2 > 0.95$ for all fitting curves.)

Similar to the results for increasing EFM thickness (Figure 29), incorporation of EFMs in composite materials extended the first 60% BSA release duration from 10 (PEGPLA hydrogels) to 24 (PCL-AAc and PCL/PEGPCL core/shell EFMs), 34 (PCL

EFMs) and 37 days (fluorinated PCL EFMs), with corresponding decreases in BSA release rate. Burst release of all seven materials was estimated as the percent of drug released after 1 day vs. M_{inf} (Table 4). Consistent with observed release kinetics (Figure 29A, 30A), burst release decreased with increasing mat thickness and hydrophobicity, which could be attributed to either increases in the diffusional path length or increased hydrophobic-hydrophilic repulsions. Additionally, the approach to constant, zero order release was roughly estimated by applying a linear fit to release data of all seven materials (Table 4). Unmodified PEGPLA hydrogels exhibited an R^2 value of 0.6331, substantially different from the near linear release of 1100 μm PCL and Fluorinated PCL composites, which exhibited R^2 values of 0.9364 and 0.9470, respectively.

Table 4. Burst effect and linear fit for hydrogel and hydrogel-EFM composites release

	PEGPLA hydrogel	300 μm PCL	800 μm PCL	1100 μm PCL	Fluorinated PCL	PCL -AAC	PCL /PEGPCL
Burst effect (%)	18.6	15.4	12.6	8.6	6.1	10.7	11.0
R^2 for linear fit	0.6331	0.7088	0.8205	0.9364	0.9470	0.7749	0.7670

Power law and modified Fick's law models were applied to further analyze early stage ($M_t/M_{inf} < 60\%$) release behavior. Comparing the power law model [325] (Eq. (4.5):

$$\frac{M_t}{M_{inf}} = kt^n \text{) and the modified Fick's law model [303] (Eq. (4.7): } \frac{M_t}{M_{inf}} = k' \sqrt{t} \text{ ,}$$

$k' = 4 \left[\frac{D_{eff}}{\pi \delta^2} \right]^{1/2}$), it can be shown that when diffusion exponent (n) is equal to 0.5, which indicates Fickian diffusion, constant k is equal to constant k' . Also, Eq. (4.7)

($k' = 4[\frac{D_{eff}}{\pi\delta^2}]^{1/2}$) shows that constant k' is proportional to $D_{eff}^{1/2}$, and inversely proportional to δ . Similar to k' , constant k depends greatly on both drug diffusivity and the characteristic length of the system. Constant k and diffusion exponent n values were obtained by fitting M_t/M_{inf} versus t data with the power law model (Figures 29A, 30A; $R^2 > 0.95$, Table 5). Constant k' was determined by evaluating the slope of the linear M_t/M_{inf} versus $t^{1/2}$ data (Figures 29B, 30B; $R^2 > 0.95$, Table 5).

First evaluating constants obtained using the power law model, it can be seen that PEGPLA hydrogels display a diffusion exponent (n) of 0.501, which indicates near Fickian diffusion. In contrast, the diffusion exponents of all composite materials investigated were > 0.5 (Fickian diffusion) and < 1 (zero-order or case II release), which denotes anomalous, non-Fickian diffusion. Diffusion exponents increased with increasing PCL EFM thickness, reflecting an approach to zero-order, linear release. Similarly, composite materials with hydrophobic EFMs (PCL and fluorinated PCL) had higher n values than those with hydrophilic EFMs (PCL-AAc and PCL/PEGPCL core/shell), nearing zero-order release as EFM hydrophobicity increased.

Constants k (power law model) and k' (modified Fickian) displayed similar trends. Composite materials displayed lower k and k' values than PEGPLA hydrogels. Values of k and k' declined with increasing mat thickness or hydrophobicity. This is consistent with Eq. (4.7) - constant k' is proportional to D_{eff} , which should decline with increasing hydrophobicity, and inversely proportional to δ , which increases with mat thickness. Taken together, these results suggest that both mat thickness and hydrophobicity contribute to changes in composite release kinetics. Unfortunately, thicker EFMs also exhibit greater hydrophobicity making it difficult to discern specific effects of increased

thickness. To deconvolute the effects of these two factors, a design of experiment factorial analysis was performed on the diffusion exponent (n). Both factors were determined to have a significant effect on n ($p < 0.004$) acting independently and together. In the experimental regions investigated (i.e., thickness ranging from 300 – 1100 μm and hydrophobicity ranging from 0 - 160° contact angle), these factors were nearly equivalent in modulating release kinetics, although superhydrophobic surfaces permitted slightly high n values to be obtained.

Table 5. Comparison of release behavior of different composite materials

	PEGPLA hydrogel	300 μm PCL	800 μm PCL	1100 μm PCL	Fluorinated PCL	PCL -AAc	PCL /PEGPCL
Water contact angle (°)	~ 0	135.2	146.5	149.6	157.0	~ 0	~ 0
Diffusion exponent n	0.501	0.519	0.538	0.623	0.692	0.537	0.516
Constant k ($\text{day}^{-1/2}$)	0.174	0.127	0.100	0.066	0.055	0.110	0.115
Constant k' ($\text{day}^{-1/2}$)	0.177	0.133	0.111	0.095	0.088	0.121	0.120

A similarly constrained system was examined by Colombo *et al.*[327], who observed that linear release could be obtained from soluble polymer matrices by holding the area of the releasing material constant, in this case by an impermeable barrier covering all surfaces but one. In contrast, our EFMs present a semi-permeable surface and therefore do not completely achieve the zero order release observed by Colombo *et al.* Instead, both EFM hydrophobicity and mat thickness serve as nearly equivalent variables, which can be tuned to alter release rate, linearity, and burst release.

4.3 Conclusions

Hydrogel drug release systems have attracted significant attention, but have not been widely implemented as a result of their large burst release and non-linear release profiles. Several groups have attempted to produce more linear profiles with reduced burst release through surface modifications, coatings, polymer physical and chemical structure modifications, non-uniform drug loading, and combination of different controlled-release systems. Here we demonstrate a composite release system, consisting of hydrophilic hydrogels and hydrophobic electrospun fiber mats in a sandwich configuration (EFM+Gel+EFM), that exhibits improved release properties. Burst release was reduced from 20% to 7%, while release duration was extended from 40 days to nearly 70 days. Additionally, release was more linear ($R^2=0.96$), resulting in more uniform drug dosing. Protein released from these composites demonstrated a slight increase in aggregation state and maintained bioactivity. Improvements in the release profile are believed to result from 1) the hydrophobic nature of the EFMs, which retards diffusion of hydrophilic drug molecules, and 2) the increased thickness and porosity of the EFM, which increase the diffusion path length.

EFM-hydrogel composites offer several advantages over hydrogel-only controlled release systems. External EFM layers in the composite material (1) significantly constrain matrix swelling in the radial direction, (2) reduce burst release, and (3) generate longer and more linear release profiles for hydrophilic therapeutics. Release behavior greatly depends on both the thickness and hydrophobicity of EFMs employed; increases in either parameter yield smaller burst release and extended, more linear release profiles. Thus,

EFM-hydrogel composites present an exciting, tunable drug release system whose properties can be easily controlled through EFM modification.

Chapter 5: Cell Adhesion to Hydrogel-Electrospun Fiber Mat Composite Materials

To improve the integration of neuroprosthetic devices with target neural tissue, PEG-hydroxyacid hydrogel-PCL-based EFM composite materials were created. These materials could not only be tuned to structurally and mechanically resemble native tissue, but also could be employed as drug delivery devices. In chapter 4, the ability of composite materials to provide controllable hydrophilic protein (e.g., neurotrophins) release was examined, and it was demonstrated that NGF release could last for ~ 3 weeks, which was close to the duration of the acute immune response to implanted devices.

However, despite the promising results of the controlled release study, to form a stable neuron-device interface, the coating materials must also be able to provide suitable surfaces for the attachment of adherent cells (e.g., neurons), a vital property for tissue regeneration. It has been reported that the interaction of adherent cells with artificial substrates can be divided into four steps: (1) cell attachment, (2) cell spreading, (3) actin cytoskeleton organization, and (4) specific focal contact formation [328]. Thus, initial cell attachment can eventually determine cell fate, including cell viability, proliferation, and function [329].

In this chapter, cell-scaffold interactions, in particular cell adhesion, are investigated using SK-N-SH (human neuroblastoma cell line) and rat cortical cell (RNC) models. SK-N-SH cell line is a mature *in vitro* model for the studies of adhesion,

apoptosis, neuroprotection, and nerve signaling of human neuronal cells [330]. Also, different from PC12 cells, SK-N-SH cells can exhibit spread, neuronal morphology with neurite extension in the absence of NGF. RNCs are directly isolated from embryonic rat cortex, and capable of forming and altering neuronal connections when they are cultured [331-333]. In addition, as primary cells, RNCs do not divide, and therefore can be cultured for months. So, RNCs provide a great cell line model in the study of neuron-composite material interaction, which is crucial in the design of biocompatible coatings for neuroprosthetic devices.

First, using an SK-N-SH cell model, we compared cell adhesion ability of six samples: PEG hydrogel, PEGPCL hydrogel, PEGPCL hydrogel-PCL EFM composite material, PEGPCL hydrogel-PCL/PEGPCL core/shell composite material, PCL EFM, and PCL/PEGPCL core/shell EFM. Samples from three categories: hydrogels, composite materials, and EFMs, were examined. PEG hydrogels, which are known for their resistance to most biological molecules adsorption and cell attachment, were studied as negative controls. To evaluate the effect of physical properties (e.g., topography, spatial architecture, and etc.) of hydrogels, composite materials, and EFMs on cell adhesion, PEGPCL hydrogel, PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite, and PCL//PEGPCL core/shell EFM were used. This experimental design ruled out the possibility of the interference from the effect of scaffold chemical cues on cell adhesion; since the chemical properties of these three scaffolds were all determined by the chemistry of PEGPCL copolymer. Then, cell adhesion to PEGPCL hydrogel-PCL EFM composite material, PEGPCL hydrogel-PCL/PEGPCL core/shell composite material,

PCL EFM, and PCL/PEGPCL core/shell EFM were compared to evaluate the impact of EFM hydrophilicity on cell attachment.

Significant improvement in cell adhesion on composite materials was observed as a result of the incorporation of external EFMs in the PEG-based hydrogel, demonstrating the efficacy of our composite materials to support cell attachment. The topographical features provided by EFMs rather than material chemical details appeared to be the primary cause of composite material cell binding enhancement. To confirm these data and demonstrate applicability in a primary cell model, rat cortical cells were examined, and this study showed that cells with higher sensitivity to the outside environment (e.g., primary cells) were likely to have a better interaction with composite materials composed of hydrophilic EFMs than the composite materials with hydrophobic EFMs.

5.1 Materials and methods

5.1.1 Effects of hydrogel-EFM composite materials on SK-N-SH cells

5.1.1.1 Preparation of samples

Diacyl-PEGPCL copolymer, and PCL, PCL/PEGPCL core/shell EFMs with a thickness of 300 μm were prepared and characterized as described in Chapter 3. For SK-N-SH cell culture experiments, 50 μL PEGPCL precursor solution was added into a well of a 96-well tissue culture plate, and then exposed to UV light for 6.5 minutes. Before precursor solution fully gelled, a piece of EFM ($D = 6\text{ mm}$) was placed in the same well, with an additional 1 minute UV exposure.

Both PBS solution and cell culture media immersions were applied to remove residual chemicals from composite materials in SK-N-SH experiments. The wells containing hydrogels were filled with 150 μL PBS solution per well and then incubated

(37°C, 5% CO₂) for one hour. Then, after PBS solution aspiration, hydrogels were immersed in cell culture media for 24 hr prior to cell seeding.

5.1.1.2 SK-N-SH cell culture

SK-N-SH cells were purchased from ATCC (ATCC, CRL-1721, Manassas, VA). Cells were cultured in growth medium (DMEM cell culture medium (Sigma-Aldrich) with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (pen-strep, Gibco)) as described in the company's protocol. First, PEG hydrogels, PEGPCL hydrogels, PEGPCL hydrogel-PCL EFM composites, PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composites, PCL EFMs and PCL/PEGPCL EFMs were deposited in 96-well tissue culture plate as described previously (N = 6 for each material). SK-N-SH cells were seeded at a density of 5×10^4 cells/cm² into each well (Figure 31) and cultured overnight in an incubator (37°C, 5% CO₂).

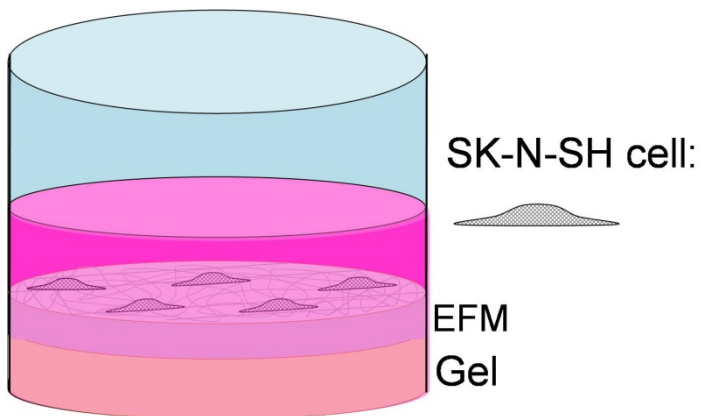


Figure 31. Scheme of SK-N-SH cells seeding on composite material samples.

5.1.1.3 Fluorescent microscopy

For fluorescent long-term cell tracking, SK-N-SH cells were pre-stained using Celltracker™ probes (Green CMFDA, Invitrogen). After 1 day incubation, fluorescently label SK-N-SH cells were imaged using a reflected differential interference contrast (DIC)

microscope (Olympus BX41) equipped with a mercury vapor lamp, fluorescent filters and with 10X and 20X objectives. Five images (per magnification) were taken at random spots for each sample (N = 3).

5.1.1.4 MTT cell adhesion and viability studies

The effect of different biomaterials on SK-N-SH cell adhesion and viability was evaluated using the MTT cell adhesion assay (N = 3), fluorescent cell long-term tracking (N = 3), and SEM imaging (N = 3). The MTT cell adhesion assay was conducted following the company's protocol (Sigma Aldrich). Since MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) can be reduced to purple formazan by enzymes in living cells; cell viability and adhesion were quantified by reading the absorbance of the sample in a Versamax UV-visible micro-plate reader.

5.1.1.5 Cell fixation and scaffold SEM imaging

SK-N-SH cells were fixed after 1 day incubation following a standard procedure. Briefly, after rinsing off residual proteins with PBS solution, cells on different samples were fixed in PBS solution containing 4% paraformaldehyde (Sigma Aldrich) and 4% sucrose (Sigma Aldrich) for 1 hour at room temperature. Then, scaffold samples were rinsed three times with PBS solution containing 4% sucrose, and dehydrated in graded ethanol (Sigma Aldrich) series and ethanol (EtOH): hexamethyldisilazane (HMDS, Sigma Aldrich) series (Table 6). After completely dehydrated, scaffolds were mounted on specimens, sputter gold coated and observed under a SEM (Quanta 200, FEI). Images of different samples were taken at magnifications of $800\times$, $1500\times$ and $3000\times$ (N = 6) and analyzed using ImageJ software (NIH) for cell spreading area and circularity assessments.

Table 6. Scaffold dehydration procedures

Components	Volumetric ratio	Dehydration time (min)
EtOH: Distilled water	50: 50	5
EtOH: Distilled water	70: 30	5
EtOH: Distilled water	80: 20	10
EtOH: Distilled water	95: 5	10
EtOH: Distilled water	100: 0	10 × 2
EtOH: HMDS	3: 1	15
EtOH: HMDS	1: 1	15
EtOH: HMDS	1: 3	15
EtOH: HMDS	0: 100	15 × 2
EtOH: HMDS	0: 100	Overnight

5.1.2 Effects of hydrogel-EFM composite materials on rat cortical cells

5.1.2.1 Preparation of samples

For rat cortical neuron culture experiments, a piece of EFM ($D = 6$ mm) was placed in a well of a 96-well cell culture cluster (Costar, Corning, NY) following the addition of 50 μ L PEGPCL precursor solution. After 6.5 minutes UV-illumination, another piece of EFM was placed in the same well. Then, composite construction was completed after 1 minute additional UV-illumination. Composite materials were secured to the bottom of a well in a 24-well tissue culture plate (2 composites per well) using sterile bio-glue (Medical adhesive silicone Type A, Dow Corning). Both PBS solution and cell culture medium immersions were also applied to remove residual chemicals from composite materials in cortical cell culture experiments.

5.1.2.2 Rat cortical cell culture

Rat cortical neurons (from E18 rat cortex) were purchased from BrainBits LLC. Since rat cortical neurons are primary cells, which are extremely sensitive to changes in environmental cues (e.g. shear force); as a positive control, poly-D-lysine hydrobromide (PDL, Invitrogen) was applied to the substrate to enhance neuron adhesion and viability. Briefly, sterile PDL solution ($0.15\text{ml}/\text{cm}^2$, $50\mu\text{g}/\text{ml}$) was placed in each well and kept for 20 hrs to permit PDL deposition. After PDL solution aspiration, the cell culture plate was rinsed with sterile $18\text{ M}\Omega$ H_2O once and dried in a sterile hood. Coated substrates were kept in a 4°C refrigerator until used.

Effects of PEGPCL hydrogel-PCL EFM composite materials and PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite materials on rat cortical neurons adhesion were studied ($N = 3$ for each sample). Hydrogel-EFMs composite materials ($N = 3$ for each sample) were sterilized and secured to the bottom of wells in PDL-coated 24-well tissue culture plate as described in Section 5.1.2.1. Rat cortical neurons were seeded at 5×10^5 cells/ cm^2 , cultured in neurobasal growth medium (Neurobasal, 2% B-27, 0.5mM Glutamax (all Invitrogen)), and incubated at 37°C , 5% CO_2 for 4 days before adhesion and viability evaluation.

5.1.2.3 MTT cell adhesion and fluorescent cell viability studies

After 4 days incubation, the MTT cell adhesion assay and a live-dead fluorescent assay were conducted to quantify rat cortical neuron adhesion on composite materials. Non-fluorescent fluorescein diacetate (FDA) can be transported across the cell membrane and converted by esterases to green fluorescent fluorescein (maximum excitation occurs at 494 nm, using a blue band pass filter), which accumulates within living cells.

Propidium iodide (PI), which is excluded from viable cells, is used for indentifying dead cells. The binding of nucleic acids to PI can enhance its red fluorescence (maximum excitation occurs at 488 nm, using a red band pass filter) and therefore identify red dead cells. Fluorescently labelled RNC cells were imaged using the same procedure described in Section 5.1.1.3.

5.1.2.4 Cell fixation and scaffold SEM imaging

After 4 days incubation of cortical cells, the same fixation, dehydration, and SEM imaging procedures as described in Section 5.1.1.5 were applied to characterize cortical cells seeded scaffolds.

5.1.3 Statistical analyses

Statistical analyses were performed using one way analyses of variance (ANOVA) with Tukey HSD testing (JMP 8.0). *p* values of < 0.05 were considered to be significant.

5.2 Results and discussions

5.2.1 Characterization of electrospun fiber mats

Water contact angles were examined to compare the hydrophobicity of different EFM surfaces. As shown in Table 7, the water contact angle of PCL EFMs was $135.2 \pm 5.4^\circ$. With the introduction of a PEGPCL shell around the PCL fiber core, the water contact angle of PCL/PEGPCL core/shell EFMs decreased dramatically to 0° . Morphology of EFMs was characterized by SEM (Figure 32). Fibers were randomly oriented in PCL and PCL/PEGPCL core/shell EFMs. Statistical analysis showed that there was no significant difference in average fiber width (PCL: 1.726 ± 0.102 vs

PCL/PEGPCL: 1.624 ± 0.058) and mean pore size (PCL: 1.517 ± 0.203 vs PCL/PEGPCL: 1.532 ± 0.194) between PCL and PCL/PEGPCL core/shell EFMs ($p > 0.05$).

Network Porosity was evaluated by the ethanol displacement method. Since porosity is defined as the ratio of the open pore volume to the total volume of scaffold; higher porosity should support better cell infiltration and transport of nutrients and waste products. The porosities of PCL EFM and PCL/PEGPCL core/shell EFM were both $\sim 82\%$.

‘Fractional contact area’, Φ , is the fraction of the fiber surface contacting other fibers. This is an important variable for EFMs, since ‘available surface fraction’, $(1-\Phi)$, is the fraction of the fiber surface which is available for cell adhesion. Eichhorn and Sampson (2005) plotted the available surface fraction $(1-\Phi)$ versus network porosity (ϵ) for networks of mean coverage ranging from two-dimensional (2D) network mean coverage to infinite (∞) [334]. The mean coverage of the real network, which is defined as the average number of fibers covering a point in a plane of a three-dimensional network formed from stacking layers of 2D networks, increases as the thickness of the network increases. Thus, based on this theory and our analysis of EFM SEM micrographs, it is reasonable to assume that the mean coverage of the EFMs with the thickness of or thicker than $300 \mu\text{m}$ is in the range of 10 to ∞ . Then, the corresponding available surface fraction $(1-\Phi)$ can be found from the figure previously reported [334]. The values of available surface fraction for PCL and PCL/PEGPCL core/shell EFMs were 0.79 and 0.73, respectively.

Table 7. Comparison of EFMs

EFM	Water contact angle (°)	Average fiber width ω (μm)	Mean Pore size (μm)	Porosity ε (%)	Available surface fraction (1- Φ)
PCL	135.2 ± 5.4	1.726 ± 0.102	1.517 ± 0.203	82 ± 2	0.79
PCL/PEGPCL	~ 0	1.624 ± 0.058	1.532 ± 0.194	82 ± 3	0.73

Data are expressed as mean $\pm$ standard deviation.

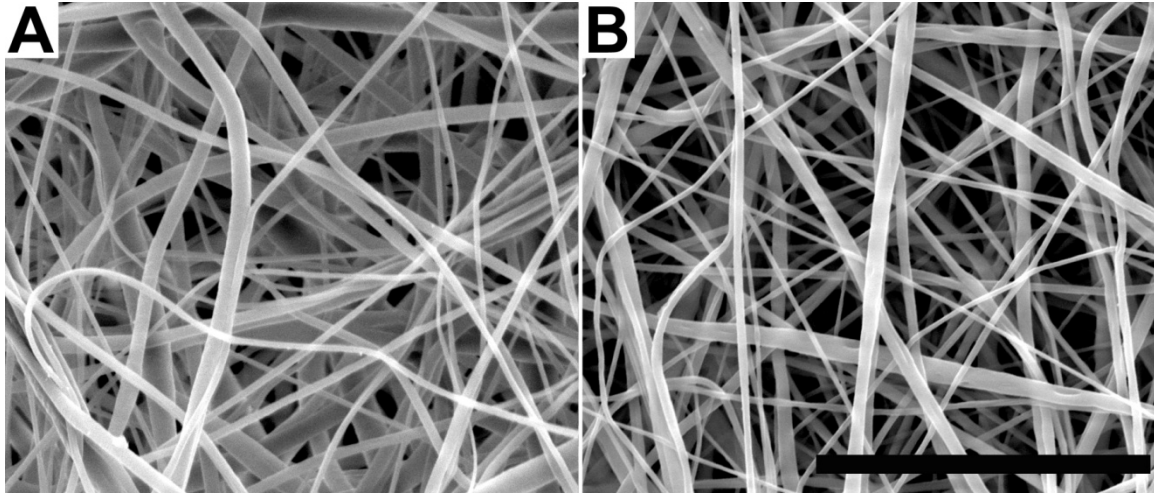


Figure 32. Scanning electron micrographs of EFMs. (A) PCL EFM; (B) PCL/PEGPCL core/shell EFM. (Scale bar: 50 μm)

The similarity of spatial architecture for both EFMs was confirmed by SEM imaging (Figure 32A, B) and thorough examination of network variables, such as fiber diameter, pore size, porosity and available surface fraction (Table 7). Whereas PCL EFMs exhibited super hydrophobicity (water contact angle $> 120^\circ$); PCL/PEGPCL core/shell EFMs were super hydrophilic (water contact angle $\sim 0^\circ$), indicating the successful incorporation of the PEGPCL shell.

5.2.2 Characterization of SK-N-SH cells adhesion to composite materials

Hydrogels, EFMs, and hydrogel-EFM composite materials were constructed to study the influence of chemical cues and physical properties of 3D matrix (such as

hydrophilicity, porosity, pore size, and topography) on cell behavior. After 1 day incubation, fluorescent labeled SK-N-SH cells were imaged using reflected DIC microscopy to compare cell attachment visually. Living cells exhibited bright green fluorescence in the micrographs. Compared with Figure 33A and 33B, which displayed the least living cells attaching to PEG or PEGPCL hydrogels, SK-N-SH cells showed significant greater adhesion to PEGPCL hydrogel-EFM composites (Figure 33C, D), and EFMs (Figure 33E, F).

Additionally, SK-N-SH cell adhesion was further quantified through the MTT cell adhesion assay. In Figure 34, the absorbance of samples with cells cultured on PEG hydrogels (A), PEGPCL hydrogels (B), PEGPCL hydrogel-PCL EFM composites (C), PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composites (D), PCL EFMs (E), PCL/PEGPCL core/shell EFMs (F) was normalized to the absorbance of samples with cells cultured on PEG hydrogels. The tested scaffolds could be divided into three categories: hydrogels, hydrogel-EFM composites, and EFMs. Significant differences in cell adhesion were found between any two of these categories ($p < 0.05$); whereas there was no significant difference in cell adhesion observed within all categories ($p > 0.05$). The results of the MTT cell adhesion assay (Figure 34) indicated that whereas EFMs were preferable for SK-N-SH cell adhesion to our composite materials and hydrogels; composite materials, despite the incorporated hydrogel material, could greatly improve cell adhesion versus hydrogels alone.

PEG is known as “stealth material” for its inertia to most biological molecules, e.g., proteins [69]. Accordingly, the results of fluorescent imaging and the MTT cell adhesion assay of SK-N-SH cells (Figure 33A and 34) demonstrated that PEG hydrogels

do not support cell adhesion. In addition, since PEG was the primary component of our synthesized PEGPCL copolymer, it is not surprising that no significant adhesion and proliferation differences for SK-N-SH cells on PEG and PEGPCL hydrogels (Figure 33 A, B and 34 ($p > 0.05$)) were observed.

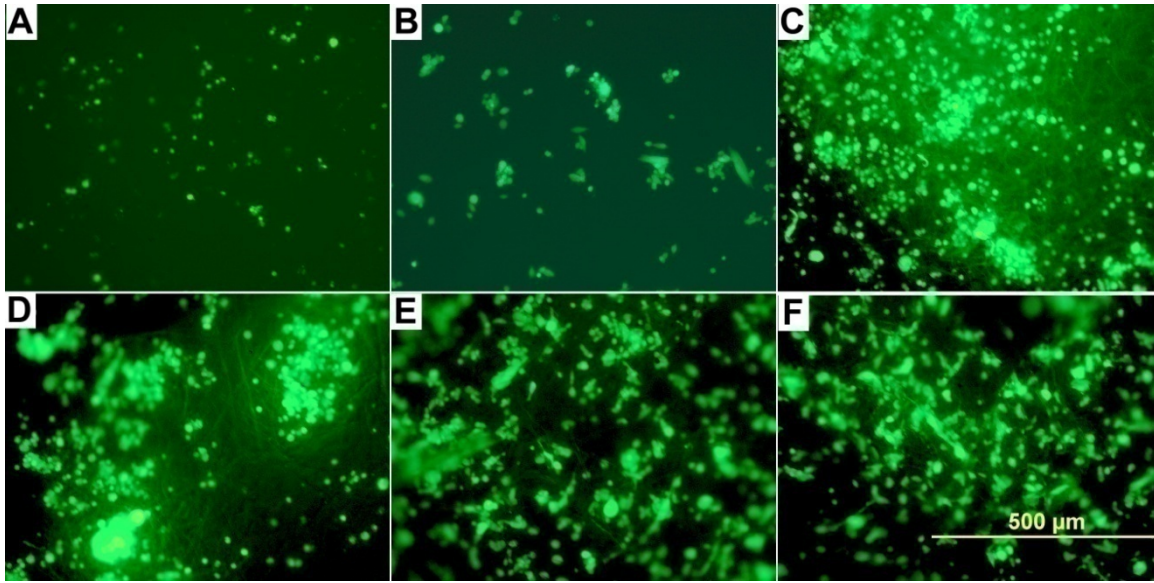


Figure 33. Reflected DIC fluorescent micrographs of SK-N-SH cells on different scaffolds, (A) PEG hydrogels; (B) PEGPCL hydrogels; (C) PEGPCL hydrogel-PCL EFM composite; (D) PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite; (E) PCL EFM; (F) PCL/PEGPCL core/shell EFM. (Scale bar: 500 μ m)

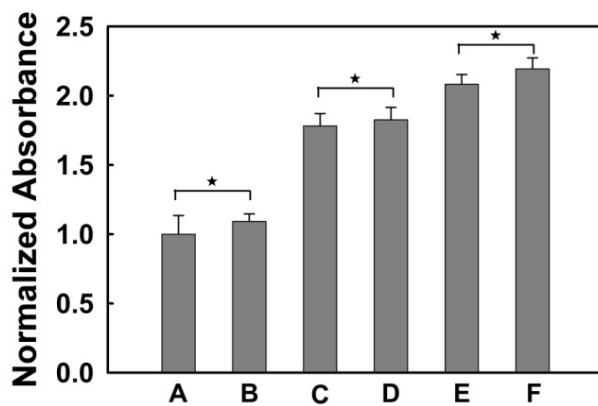


Figure 34. SK-N-SH cell adhesion on different scaffolds, (A) PEG hydrogels; (B) PEGPCL hydrogels; (C) PEGPCL hydrogel-PCL EFM composite; (D) PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite; (E) PCL EFM; (F) PCL/PEGPCL core/shell EFM. (Asterisks indicate no significant difference between two samples ($p > 0.05$))

In addition, SK-N-SH cells displayed significant different adhesion to the scaffolds (PEGPCL hydrogels < PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite < PCL/PEGPCL core/shell EFM) (Figure 34). Since the surface chemical properties of PEGPCL hydrogels, PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite, and PCL/PEGPCL core/shell EFM were all determined by the chemistry of PEGPCL copolymer; the differences of SK-N-SH cell adhesion to these samples probably resulted from the differences of the physical properties (e.g., topography and 3D architecture) of hydrogels, composite materials, and EFMs.

To obtain detailed information on SK-N-SH cell morphology, scanning electron micrographs of hydrogel-EFM composite and EFM samples were taken, as shown in Figure 35. In all SEM images, cells spread on EFMs and interacted simultaneously with multiple fibers. However, although SK-N-SH cells cultured on all samples displayed the tendency to spread along contacting fibers; cells adhered to composite materials kept relatively round shapes (Figure 35C, D), indicating a less favorable interaction between SK-N-SH cells and composite materials than the interaction between cells and EFMs. These results were consistent with the results of the MTT cell adhesion study, which showed that SK-N-SH cell adhesion/proliferation on hydrogel-EFM composites was significant lower than that on EFMs alone (Figure 34).

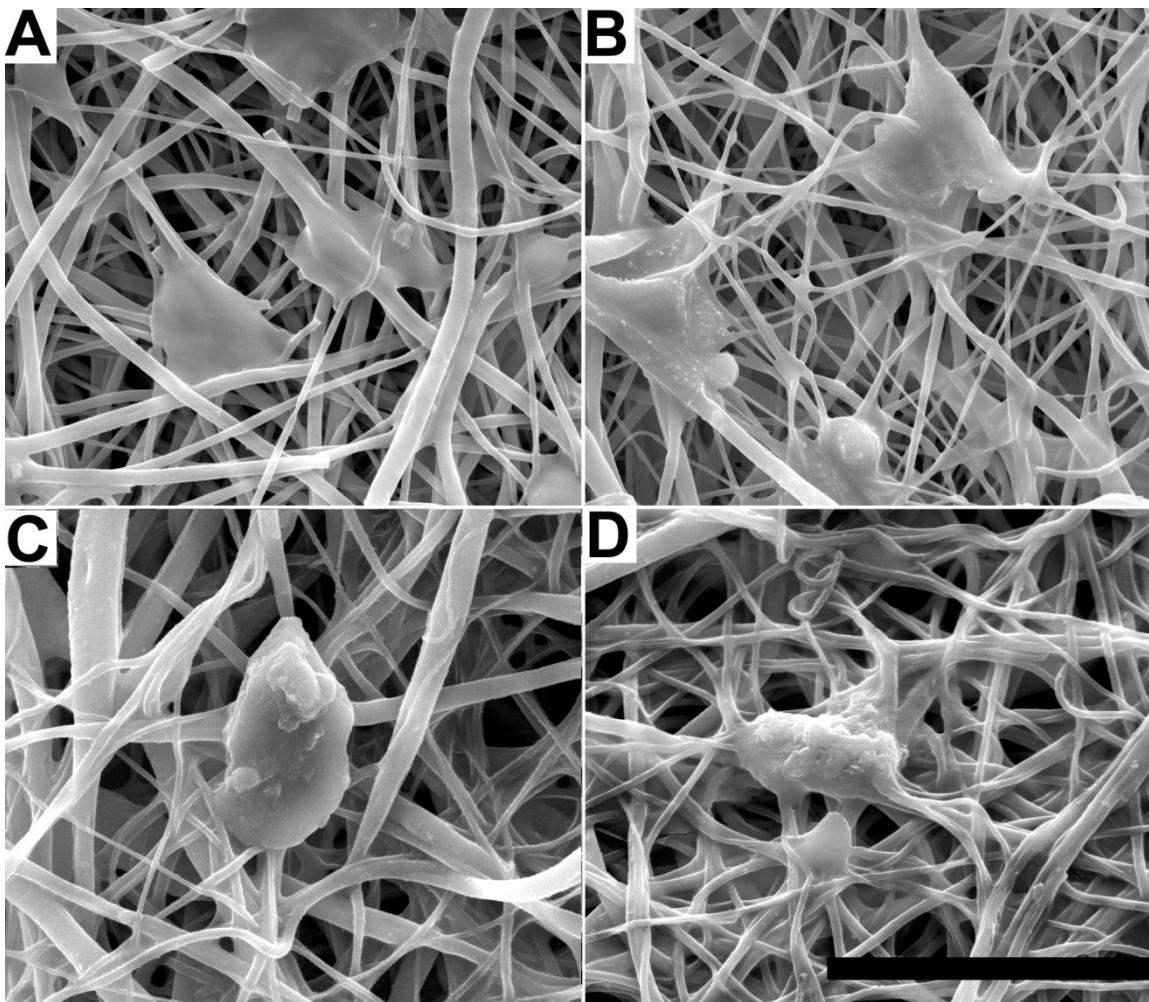


Figure 35. SEM images of SK-N-SH cells on different scaffolds, (A) PCL EFM; (B) PCL/PEGPCL core/shell EFM; (C) PEGPCL hydrogel-PCL EFM composite; (D) PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite. (Scale bar: 50 μ m)

This most likely results from PEGPCL hydrogel layer penetration into the incorporated EFM layers, resulting in remarkable changes in scaffold 2D surface features such as edges, grooves, steps, and pore interconnectivity. Also, the interaction and infiltration between EFM and hydrogel probably changed not only the roughness of EFM surfaces, but also the mean pore size and porosity of EFMs. As reported previously [335], different types of cells may require specific “optimum pore size ranges” to maximize cell

infiltration. So, besides affecting cell adhesion and migration [336], altered pore sizes could preferentially promote or exclude the ingrowth of specific types of cells [337].

Cell spreading area and circularity were analyzed using measure tools in Image J software. Although the average values of cell spreading area for PCL EFM and hydrogel-PCL EFM samples were higher than those of PEGPCL/PCL core/shell EFM and hydrogel- PEGPCL/PCL core/shell EFM samples; there was no significant difference in cell spreading area for all samples ($p > 0.05$) (Figure 36) and additional experiments will be needed to confirm this finding. Circularity of SK-N-SH cells cultured on EFM scaffolds (PCL and PCL/PEGPCL core/shell) was significant lower than that of cells on hydrogel-EFM composite scaffolds ($p < 0.05$) (Figure 37), in accordance with the cell morphology observed under SEM (Figure 35). In a word, although no significant differences in cell spreading area was shown ($p > 0.05$) (Figure 36); cells cultured on EFM samples displayed lower circularity than those on composite material samples (Figure 37), which confirmed the effect of substrate surface texture on cell spreading [338].

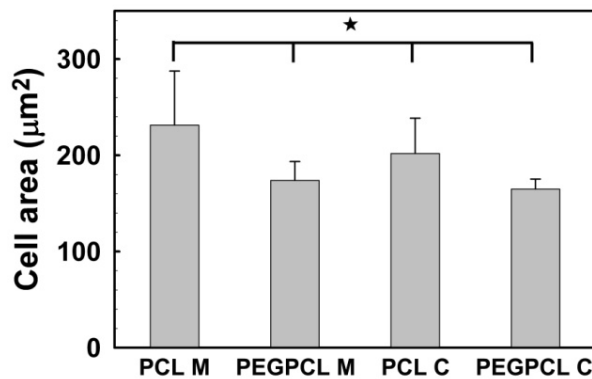


Figure 36. Spreading area of SK-N-SH cells on different scaffolds, from left to right: PCL EFM; PCL/PEGPCL core/shell EFM; PEGPCL hydrogel-PCL EFM composite; PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite. (Asterisk indicates no significant difference between pair samples)

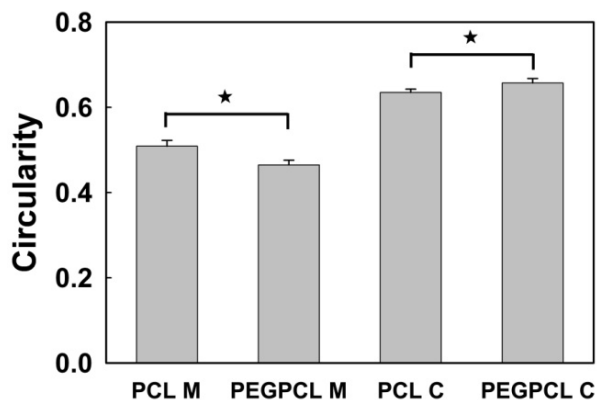


Figure 37. Circularity of SK-N-SH cells on different scaffolds, from left to right: PCL EFM; PCL/PEGPCL core/shell EFM; PEGPCL hydrogel-PCL EFM composite; PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite. (Asterisks indicate no significant difference between two samples)

Although PCL and PCL/PEGPCL core/shell EFMs displayed completely different hydrophilicity, there was no significant difference in either adhesion (Figure 33E, F, and 34) or morphology (Figure 35C, D, 36 and 37) of SK-N-SH cells cultured on these EFMs. This finding was in accordance with the results reported by Curtis and Wilkinson (1999) [339], who claimed that the primary cause of cell “topographic reactions” (cell adhesion, cell shape, and a series of intracellular biochemical reactions, such as activation of tyrosine kinases and control of gene expression) to material surfaces is the cell stretch reaction to the substrate instead of the cell response to chemical stimuli of the substrate. Additionally, our results also indicated that the change in hydrophilicity had only a slight influence on SK-N-SH cell behavior, which was different from some existing reports. This is probably due to the fact that different types of cells could exhibit significantly different sensitivity to scaffold hydrophilicity. Thus, the results of SK-N-SH cell adhesion studies confirmed that scaffold spatial architecture (e.g., topographical and mechanical properties) were the dominant variable for cell “topographic reaction”.

5.2.3 Characterization of rat cortical cells on composite materials

Following SK-N-SH cell studies, the interaction between rat cortical cells and composite materials was investigated. The results of the MTT cell adhesion assay showed that cortical cells cultured on PEGPCL hydrogel-PCL EFMs, PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite materials, and positive control samples displayed similar low cell attachment. It was not surprising to observe relatively low viability and adhesion in primary cells like cortical cells; since they are extremely vulnerable and sensitive to changes of environmental cues (e.g., shear force, temperature, CO₂ concentration, and etc.), and usually require the addition of specific growth factors in cell culture medium to enhance their survival rate. Also, these results were consistent with the manufacturer's protocol, indicating that the average viability of this cell line is around 20% after thawing. Fluorescent images of cortical cells on PDL coated substrate (positive control) are shown in Figure 38. Healthy cells (Figure 38A) not only exhibited larger volumes when compared with dead or apoptosing cells (Figure 38B), but also displayed evident neurite extension.

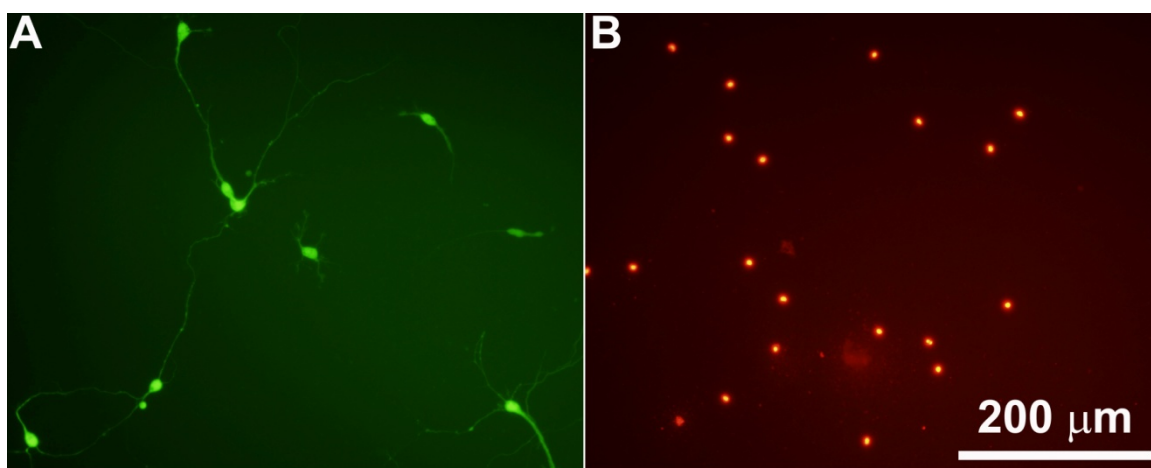


Figure 38. Fluorescent micrographs of cortical cells on PDL coated substrates, (A) fluorescein-label living cells, (B) propidium iodide (PI)-label dead cells.

Next, the morphology of cortical cells cultured on composite materials was characterized by SEM, as shown in Figure 39. It appeared that cells on PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite samples exhibited a more spread morphology, indicating a better interaction between cortical cells and composite materials comprised of hydrophilic EFMs.

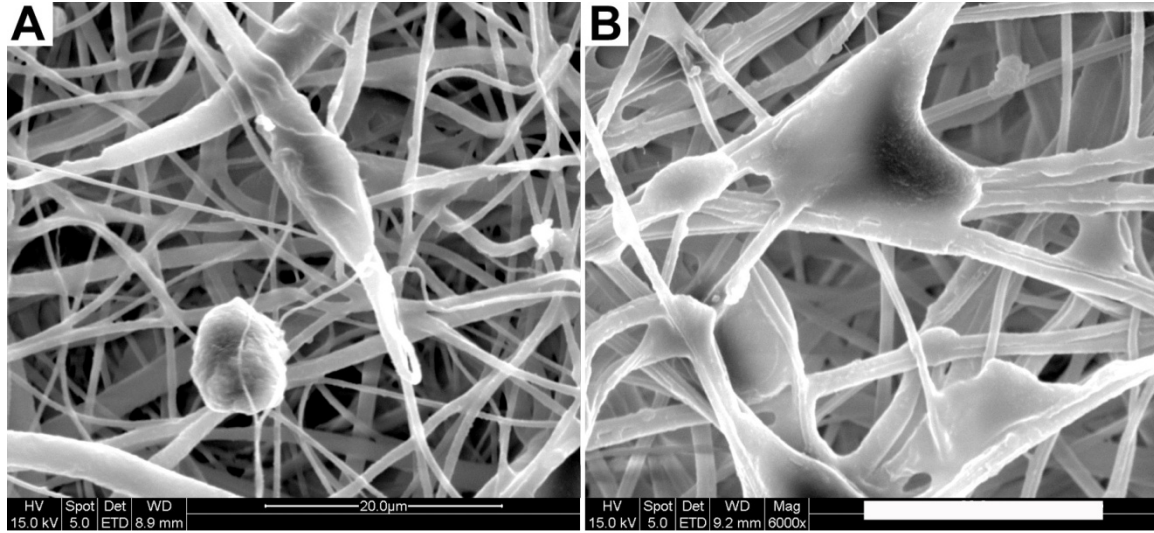


Figure 39. SEM images of rat cortical cells on different scaffolds, (A) PEGPCL hydrogel-PCL EFM composite; (B) PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite. (Scale bar: 20µm)

To verify this hypothesis, cell spreading area and circularity were evaluated using Image J software. The results showed that cells on PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite materials had significantly larger spreading areas than those cultured on PEGPCL hydrogel-PCL EFM composite materials ($p < 0.05$) (Figure 40). However, no significant difference in cell circularity was found between these two samples ($p > 0.05$) (Figure 40). Thus, compared with the results of SK-N-SH cell studies where hydrophilicity of materials (substrates) had no significant impact on cell spreading area (Figure 36), the hydrophilicity of the incorporated external EFM significantly changed cortical cell spreading area. This finding confirmed our previous hypothesis that

cell lines with different sensitivity to environmental stimuli could respond differently to changes in scaffold hydrophilicity.

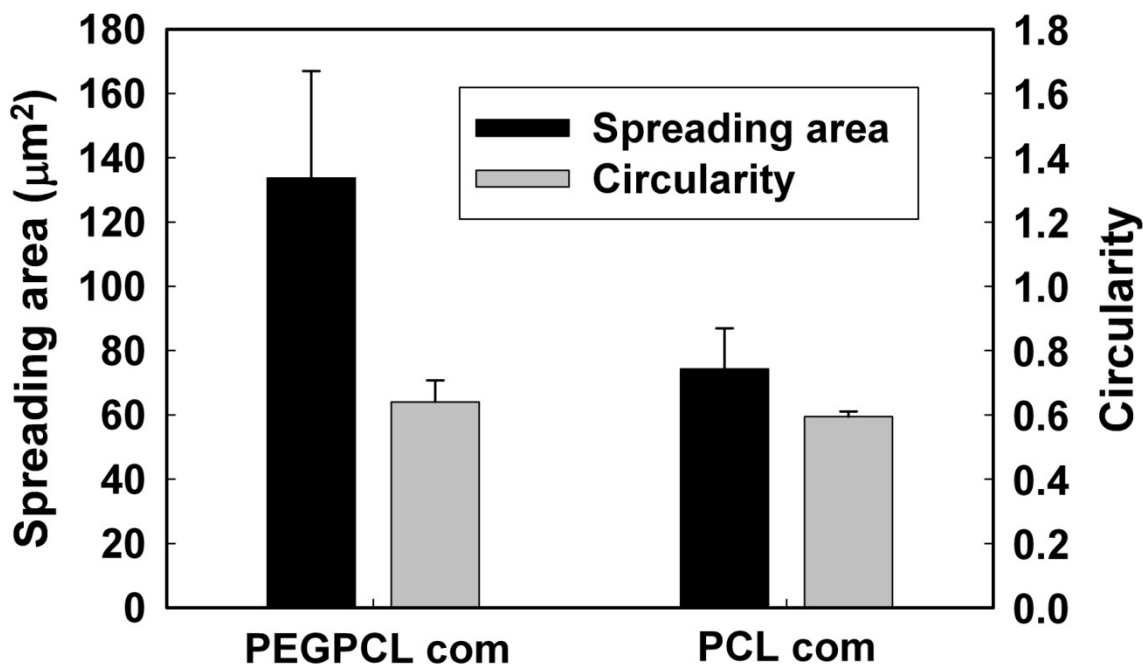


Figure 40. Spreading area and circularity of cortical cells on different scaffolds, from left to right: PEGPCL hydrogel- PCL/PEGPCL core/shell EFM composite, and PEGPCL hydrogel- PCL EFM composite.

5.3 Conclusions

These studies of SK-N-SH and rat cortical cells demonstrate the great potential of hydrogel-EFM composite materials in promoting cell adhesion, and therefore improving the integration of target neural tissue with implanted devices. It is believed that scaffold spatial architecture (e.g., topographical and mechanical properties) plays a fundamental role in control of SK-N-SH cell adhesion. In addition, for rat cortical cells, biomaterial chemical properties, such as hydrophilicity, also had important impact on cell behavior, including cell attachment and morphology.

In the future, to obtain better cell-matrix interaction, advanced EFM fabrication techniques could be applied to introduce biological cues into composite materials, such as coating the EFM surface with adhesion molecules or co-electrospinning PCL with natural polymers derived from ECM. Also, cell infiltration into composite materials is important for the formation of stable tissue-device interfaces. More experiments should be performed to optimize material spatial architecture (e.g., EFM pore size and porosity, hydrogel crosslinking density and mesh size.) for cell infiltration. Thus, our composite materials showed great potential in tissue regeneration, and could be tuned to meet the requirements of different biomedical applications.

Chapter 6: Hydrogel-Electrospun Fiber Mat Composite

Materials as Coatings at the Neuroprosthetic Interface³

Neural prostheses have been explored for over two decades for their potential to restore lost function in the CNS [1, 24, 26, 28]. However, clinical applications of these devices have been limited by the long term instability of the CNS tissue-device interface [11, 340]. As described in Section 1.2, two primary factors that compromise electrode efficiency are 1) local tissue anatomy, which significantly separates electrodes from target neurons [27, 28], and 2) glial scar formation [29-31]. These limitations ultimately lead to an increase in electrode impedance, which in turn affects device performance. Thus, to reduce the reactive response to implanted devices, considerable efforts have been made to improve the biocompatibility of neural prostheses, including modifications of the electrode material, electrode shape, and implantation techniques [10, 27, 49], application of drugs to minimize reactive gliosis [57, 59, 60], and application of electrode coatings such as hydrogels [57, 60, 341], layer-by-layer films [56, 63, 342], and conducting polymers [55, 64, 343].

Because of their potential to promote neuronal survival and growth, neurotrophins have been widely used in nerve regeneration [326, 344]. By incorporating neurotrophins (e.g. NGF) into electrode coatings for controlled release to surrounding tissue, target neurons can be stimulated to survive and extend neurites toward electrode sites. This

³ Portions of this material have been submitted to *Frontiers in Neuroengineering*.

should reduce the separation distance between target neurons and electrodes. Unfortunately, the extremely short *in vivo* half-lives of neurotrophins make their sustained release challenging [345, 346]. Previously, we demonstrated the potential of neurotrophin-eluting PEGPLA hydrogels as coatings for planar microelectrode arrays (MEAs) [57]. Photo-polymerized, biodegradable, PEG-based hydrogels were selected for their biocompatibility [347, 348], ability to gel *in situ* [83, 349], and facile tailoring of release characteristics by changing polymer chain length, degradable repeat ratio, and cross-linking density [82]. We showed that PEGPLA hydrogel coatings persisted on electrode surfaces for ~ 7 days in PBS immersion bath and ~ 11 days under an agarose gel, and released bioactive NGF for at least 9 days [57]. Although these results were promising in terms of reducing early reactive response caused by the trauma of electrode implantation (3~10 days post-implantation) [31]; coatings capable of longer electrode adhesion and neurotrophin elution are required to address the sustained reactive response, which is believed to occur after 2 weeks post-implantation and last for at least 2 months [31].

In this chapter, we present hydrogel-EFM composite electrode coatings, which adhered to electrode surfaces for over 9 months and eluted neurotrophins for at least 25 days. Since the duration of drug release and coating adhesion on electrodes can be increased by reducing polymer degradation rate [82, 292], PEGPCL hydrogels were used in the composite coating instead of comparative molecular weight PEGPLA because of their slower degradation rate [322, 323]. PCL hydrophobic EFMs were used to construct hydrogel-EFM composite coatings because of their excellent biocompatibility and low biodegradation rate [350]. These novel hydrogel-EFM composites combine the appealing

features of both hydrogel and EFM systems, and hold great potential to improve the biocompatibility of implanted devices, thereby enhancing chronic electrode performance.

6.1 Materials and methods

6.1.1 Formation of hydrogel-EFM composite material coatings on multi-electrode arrays

6.1.1.1 Multi-electrode arrays

Microelectrode arrays (MEAs) were generously provided by Dr. Stuart Cogan (EIC Laboratories, Norwood, MA), as shown in Figure 41. The arrays are composed of 15 (3×5), 400 μm circular, gold electrodes covered by a sputtered 300nm film of iridium oxide (SIROF) and a 50nm titanium film between the electrode sites and SIROF, fabricated on a $\sim 10\mu\text{m}$ thick polyimide substrate [351].

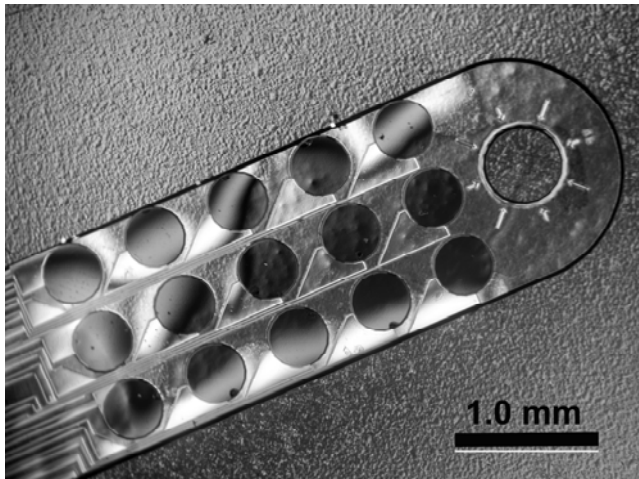


Figure 41. Optical micrograph of the distal tip of an MEA.

6.1.1.2 Formation of composite material coatings on MEAs

PEGPCL hydrogel-PCL EFM composites were created via photo-polymerization as described in previous chapters, following the method of Hubbell et al. [82]. Briefly,

precursor solution was prepared by adding initiators (Irgacure 2959, 0.1wt%, Ciba, Tarrytown, NY, and 1-vinyl-2-pyrrolidone, 0.5 wt%) into a solution of 22wt% diacryl-PEGPCL polymer in D-PBS. 2 μ L of precursor solution was placed on the desired substrate (i.e., TeflonTM for Eluted NGF measurement exp., electrode arrays for electrode coating adhesion, biocompatibility, and eluted NGF bioactivity tests), and exposed to UV illumination (Blak-Ray, B-100-AP, UVP) for 2 min to form PEGPCL hydrogel boluses. For composites formed on TeflonTM (shown in Figure 42A), PCL mats (D = 1.5 mm) were placed on TeflonTM, followed by the deposition of 2 μ L PEGPCL precursor solution on top of each mat, and exposed to UV illumination for 90 s. A second PCL mat was then placed on top of each bolus, and exposed to UV illumination for an additional 45 s. For composite electrode coating formation (shown in Figure 42B), 2 μ L of PEGPCL precursor solution was dispensed over the distal end of a 3 $\times$ 5 electrode array, exposed to UV illumination for 90 s, followed by addition of a PCL mat (D = 1.5 mm) to the top of the bolus and continuing UV exposure for another 45 s.

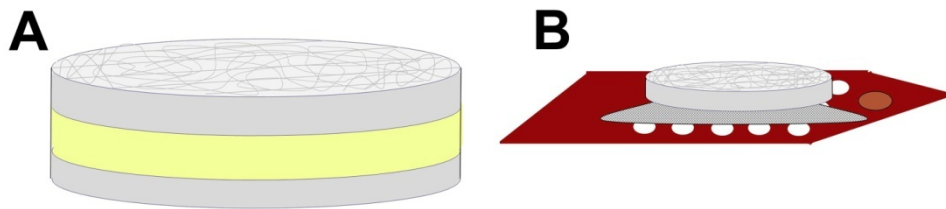


Figure 42. Schematic of (A) hydrogel-EFM composite material, gray = EFM, yellow= hydrogel; (B) composite coating on an MEA.

6.1.2 Electrode coating adhesion studies

6.1.2.1 Implantation studies

PEGPCL hydrogel-PCL EFM composites were deposited as electrode array coatings through UV illumination as described in Section 6.1.1.2 (Figure 42B). The

coated electrode array was then inserted into an agarose gel tissue phantom (1% wt/v in D-PBS) and removed immediately. The integrity of coating was visually evaluated by phase contrast microscopy at $10\times$ and $20\times$ magnifications.

6.1.2.2 Phosphate buffer saline bath immersion

Coated MEAs were immersed in a PBS bath (10 mL PBS in 15 mL centrifuge tube). Long term composite coating adhesion (up to 9 months in D-PBS solution) (N=2 coated electrodes) was assessed once a month by reflected differential interference contrast optical microscopy (Olympus BX41) and SEM (Quanta 200, FEI).

6.1.2.3 Agarose gel phantom tests

Additionally, coated electrode arrays were placed under an agarose gel tissue phantom (1 % wt/v in D-PBS), which mimics the tissue that would constrain composite coatings under *in vivo* conditions. Coating adhesion was evaluated by the use of inverted phase contrast microscopy (Olympus IX71) at $10\times$ and $20\times$ magnifications over a month. All images were converted to grayscale using Adobe Photoshop (Version 10.0).

6.1.3 Efficiency of composite material coatings

6.1.3.1 Biocompatibility

Before utilization in cell culture and NGF elution experiments, PEGPCL precursor solution was sterile-filtered through a $0.22\ \mu\text{m}$ syringe filter with a $0.8\ \mu\text{m}$ pre-filter (Millipore). Biocompatibility of composite electrode coatings was evaluated by culturing PC12 cells (ATCC, CRL-1721, Manassas, VA) at 1×10^4 cells/cm² in collagen-coated 24-well tissue-culture plates. Cells were incubated at 37°C, 5% CO₂ overnight in 0.5 mL standard culture medium (Ham's F12K Medium with 2mM L-glutamine, 1.5 g/L sodium bicarbonate, 2.5% fetal bovine serum, 15% horse serum (all from Sigma-Aldrich),

and 1% penicillin-streptomycin (Gibco, Carlsbad, CA)). Three different samples (Figure 43) were evaluated in addition to a control sample (PC12 cells in culture wells). *Sample C*: Hydrogel-EFM composites (N=3) of 2 μ L hydrogel phase were created on TeflonTM and placed in a transwell insert. *Sample EC*: a composite-coated electrode array was attached to the side wall of a well using adhesive carbon tape to avoid direct contact with PC12 cells at the bottom of the well. *Sample ECB*: an electrode array coated by composite containing 5 wt% bovine serum albumin (BSA, model drug) in the hydrogel phase was attached to the side wall of a well. Cells were cultured in the presence of samples (N = 3 for each sample) for 2 weeks and then analyzed by the MTT cell viability assay following the manufacturer's instructions. Data was collected using a Versamax UV-visible micro-plate reader with measurements performed in triplicate. Pooled data from each repetition was analyzed using one way ANOVA ($\alpha = 0.01$). Pair-wise comparisons were performed using the Holm-Sidak method ($\alpha = 0.05$).

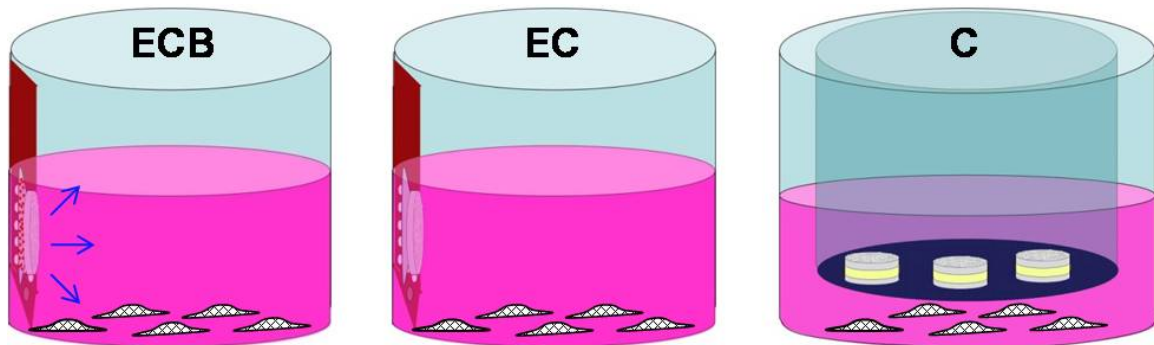


Figure 43. Schematics of biocompatibility tests: sample ECB; sample EC; sample C.

6.1.3.2 Elution of NGF from composite material coatings

For NGF elution experiments, sterile PEGPCL precursor solution was added to lyophilized NGF (2.5 S, Promega, Madison, WI) at a concentration of 1 μ g/ μ L. Hydrogel-EFM composites (N=3) with a 2 μ L NGF-containing hydrogel phase were

created on TeflonTM as described in section 6.1.1.2 (Figure 42A), and placed on the upper surface of 0.4 μm membrane transwell inserts (Corning) (Figure 44). The insert was then placed in a 24-well tissue-culture plate (BD Biosciences) filled with 0.5 mL D-PBS. To determine the amount of eluted NGF, 450 μL samples ($N = 3$) were collected at each time point (up to 25 days) and measured using the NGF E_{Max} ELISA assay (Promega), following the manufacturer's instructions for each sample. Fresh D-PBS was then used to replace the sample collected.

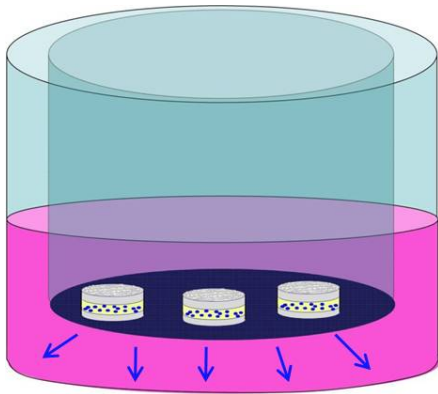


Figure 44. NGF elution from composite materials.

6.1.3.3 Study of bioactivity of NGF released from coatings in a PC12 cell culture model

Similar to the bioactivity study of eluted NGF in Section 4.1.1.5, PC12 cells were used because of their ability to exhibit a neuronal phenotype including neurite extensions upon exposure to NGF, and retract neurites after NGF is withdrawn from the culture media. In the absence of NGF, PC12 cells maintain a rounded morphology with few neurite extensions. Cells were cultured at 1×10^4 cells/cm² in 24-well plates overnight. Electrode arrays coated with composites containing 5wt% BSA or 1 μg NGF/ μL in the hydrogel phase were attached to the side walls of wells (one electrode array/well,

triplicate for each sample) [Note that cells do not contact the composite coatings directly] (Figure 45). Cells were cultured for two weeks with medium exchanged every 2-3 days. Samples were compared to two controls, a positive control (N = 3) which received 50 ng/mL NGF added directly to the medium and a negative control (N = 3) which received no NGF. Neurite extension was assessed using phase contrast microscopy (Olympus IX71) at 10 $\times$ and 20 $\times$ magnifications at day 7 and day 14 for all samples. Five pictures were randomly taken for each well. Neurite length (from the tip of each neurite branch to the soma) was measured using Image J software. Kruskal-Wallis one-way analysis of variance by ranks ($\alpha = 0.01$, for non-parametric data) was applied to analyze pooled data from each repetition, whereas the Tukey test ($\alpha = 0.05$) was performed for pair-wise comparisons.

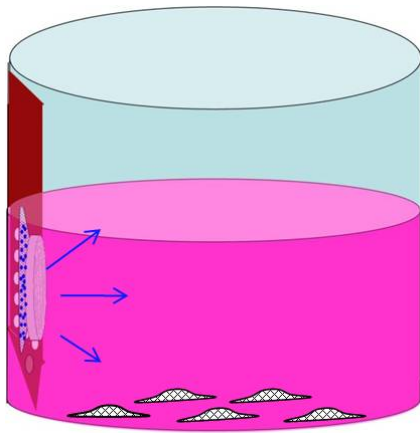


Figure 45. Schematic of the NGF bioactivity study.

6.2 Results and discussions

6.2.1 Characterization of composite coating electrode adhesion

PEGPCL hydrogels and PEGPCL hydrogel-PCL EFM composites adhere to electrode arrays as shown in short-term optical micrographs (Figure 46A, B) and SEM

(Figure 46C). The interaction of PCL EFMs with PEGPCL hydrogels can be seen by the roughness of the coating surface near the PCL EFM in Figure 46C, which probably resulted from the non-uniform shrinkage of the hydrogel as it was constrained by the EFM during dehydration. Coatings demonstrate both short and long term adhesion on electrode surfaces in the presence of PBS (Figure 46D, E, 45 days and Figure 46F, 9 months). In Figure 46D and E, the irregular margin outside the original hydrogel-EFM composite interface indicates the perimeter of degraded hydrogel. In contrast with Figure 46D, the degraded hydrogel film in Figure 46F has broken into small pieces covering individual electrode sites. Although degradation of the PEGPCL hydrogel component is evidently advancing in Figure 46D, E and F as seen by the receding edge of hydrogels, we also observed that coating regions not covered by EFMs (i.e., PEGPCL hydrogel only) degraded much more rapidly than regions covered by PCL EFMs (Figure 46E, F). These results suggest that PCL EFMs physically constrain the hydrogel bolus and increase the water diffusion barrier, thereby significantly reducing hydrogel degradation and improving coating adhesion duration. It is thus possible that adhesion duration of PEGPCL hydrogel-PCL EFM composite electrode coatings could be controlled by applying EFMs of different shapes and sizes. Additionally, some wrinkling of the EFM is evident in Figure 46F, likely driven by shrinkage of the hydrogel layer beneath it. Composite coatings demonstrated adhesion for at least one month in a tissue phantom test, in which a coated electrode array was placed under an agarose gel mimicking the effect of tissue (Figure 46G).

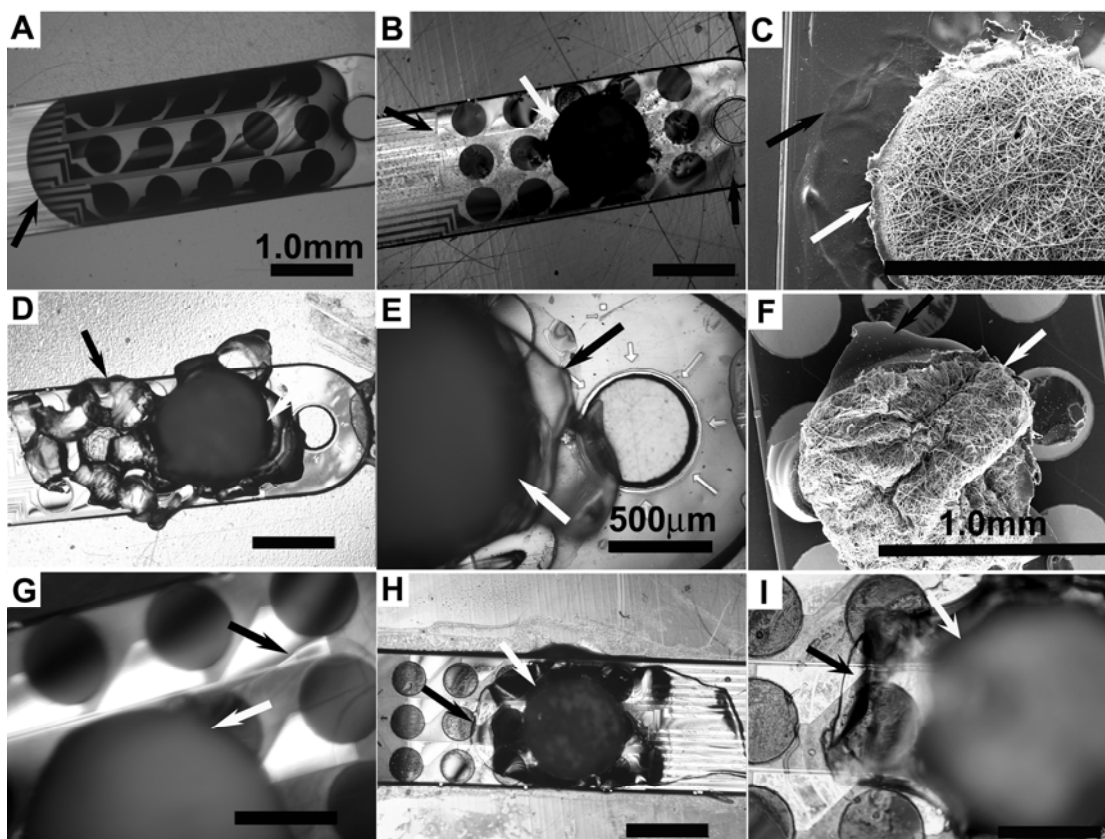


Figure 46. Micrographs of PEGPCL hydrogel-PCL EFM adhesion to electrode array surfaces. (A) Optical micrographs of the distal tip of an MEA array coated with PEGPCL hydrogel and (B) coated with PEGPCL hydrogel-PCL EFM composite at day 0. (C) SEM micrograph of the distal tip of an MEA coated with PEGPCL hydrogel-PCL EFM composite at day 0. (D) and (E) Optical micrographs of composite coatings after soaking in PBS buffer for 45 days. (F) SEM micrograph of composite coating after soaking in PBS solution for 9 months. (G) Optical micrograph of composite coating at day 32 under an agarose tissue phantom. (H) and (I) Optical micrographs of composite + electrode array after insertion into and removal from an agarose tissue phantom. (Black arrows indicate the edge of the PEGPCL hydrogel; white arrows indicate the edge of PCL EFMs. Scale bar: 1 mm for A, B, C, D, F and H; 500 μ m for E, G and I.)

Compared to the PEGPCL [287] and PEGPLA [57] hydrogel coatings investigated previously, which adhere to the electrode array surface for at least 28 and 10 days, respectively, PEGPCL hydrogel-PCL EFM composite coatings displayed significantly longer electrode adhesion (over 9 months in PBS bath immersion tests (Figure 46F) and over 1 month in tissue phantom tests (Figure 46G)).

Apart from coating adhesion, we also investigated the potential of coatings to withstand implantation into the brain. Each of the three composite electrode coatings was inserted into a 1% wt/v agarose gel (brain tissue has similar mechanical properties to softer 0.6% wt/v agarose gels [352]). No delamination was observed for the coatings, demonstrating their potential to withstand the physical stresses associated with *in vivo* implantation (Figure 46H, I). These results suggest that composite coatings are able to withstand compression or shearing forces encountered during implantation, and the constraint created by PCL EFMs increases the persistence of the composite coating on the electrode surface and its potential for resisting implantation forces in clinical application. An interesting possibility is that the stability imparted by EFMs to the coatings may provide additional resistance to device movement associated with use, a target for future research.

6.2.2 Biocompatibility of composite electrode coatings

PEGPCL hydrogel-PCL EFM composite electrode coatings did not demonstrate evident toxicity in a PC12 cell culture model. An MTT assay (Figure 47) indicated that negative control samples (PC12 cells cultured without exposure to composites or composite electrode coatings) demonstrate no significant difference in cell number compared with samples exposed to composites (C), composite electrode coatings (EC), and composite electrode coatings releasing BSA (ECB) ($p = 0.81$, $N = 3$, average of three replicates). Also, in phase contrast optical micrographs, cells responded to C, EC, and ECB by maintaining their undifferentiated, rounded morphology (Figure 48). No qualitative morphology change was observed in all samples. Since hydrogel-EFM composites are comprised of biocompatible materials, PEGPCL and PCL, which have

been extensively used in the clinic without evident cytotoxicity [353], it is not surprising to find that composite electrode coatings are biocompatible and can be further used as controlled neurotrophin delivery vehicles.

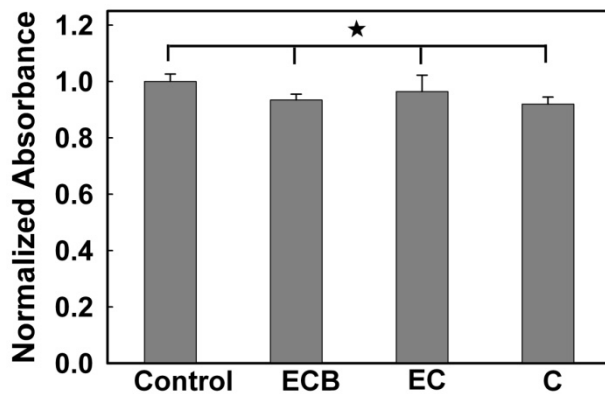


Figure 47. Normalized absorbance from an MTT cell viability test. (The asterisk indicates no significant difference ($p > 0.05$))

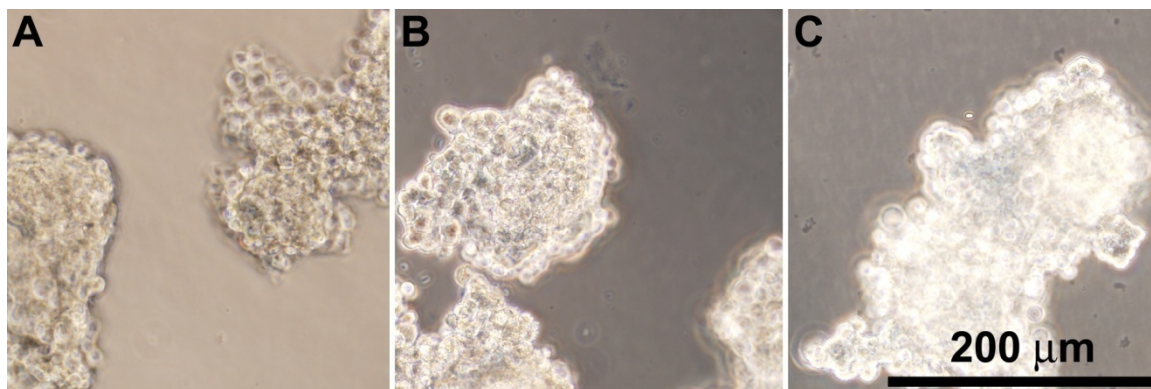


Figure 48. Representative phase contrast optical micrographs of PC12 cells after 14 days cell culture. (A) Sample C; (B) Sample EC; (C) Sample ECB as shown in Figure 43.

Another important consideration in composite coating biocompatibility is size, since it is crucial to minimize the total size of neural prostheses, including coatings, to reduce device-related tissue damage. The thickness of hydrogel-EFM composites is easily controlled. By changing the weight percentage of polymer solution and the volume

of the hydrogel bolus, we can modify the thickness of the PEGPCL hydrogel layer. The application of PCL EFMs on top of the hydrogel layer can improve the uniformity of the hydrogel coating, and the thicknesses of PCL EFMs can be easily modified by changing electrospinning fabrication parameters, i.e., polymer solution flow rate, electrospinning deposition time, and tip-to-substrate distance.

Although electrochemical properties of composite electrode coatings were not characterized through cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), and potential transient measurements, the results of previous electrochemical characterization [57] showed very little effect of PEGPLA hydrogel coatings on the charge-injection properties of coated electrodes, and no significant difference of the maximum electrochemical potential excursions and access voltage between coated and uncoated electrodes. Since PEGPCL hydrogels have similar physical and electrochemical properties to PEGPLA hydrogels, we believe it is very unlikely that a significant difference in electrochemical properties would be observed. Also, the effect of the coating on the electrode electrical properties would be mitigated by continued polymer degradation. Furthermore, to fully expose the electrode sites to surrounding tissue without any compromise of their electrical properties, patterning techniques such as photolithography [90] or microfluidic channels [354] can be applied to control the spatial properties of the hydrogel coatings. Similarly, to avoid the potential influence of composite EFMs on electrode electrochemical properties, the shape of EFMs can be tailored to the pattern of electrode array, exposing the electrode sites to surrounding tissue. Also, laser ablation [355] or photolithography [356] can be applied to modify

EFMs to desired pattern. In this way, electrode sites could be exposed while the rest of the MEA could be covered by coatings.

6.2.3 Measurement of eluted NGF

The NGF $E_{\max}$ ELISA assay was used to determine the quantity of NGF eluted from PEGPCL hydrogels and PEGPCL hydrogel-PCL EFM composites. NGF was released from all samples over a period of 25 days (Figure 49), near our target of 4 weeks, demonstrating their potential for future *in vivo* applications.

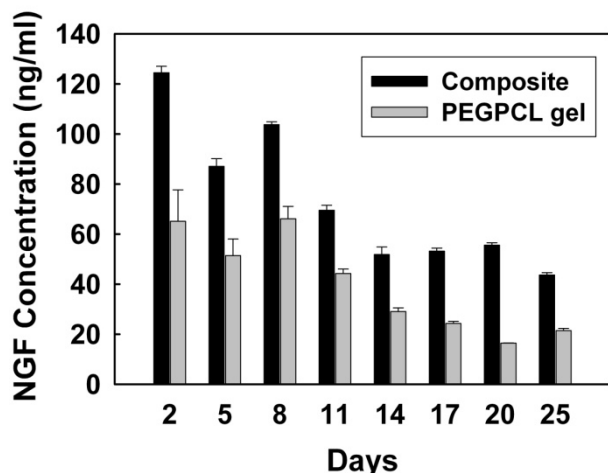


Figure 49. Concentration of eluted NGF as measured by ELISA.

The concentration of NGF released from composite materials was higher than that of PEGPCL hydrogels at each time point, despite equal initial NGF loading. After 25 days exposure, composite materials showed significantly extended release of NGF vs. PEGPCL hydrogel controls. The concentration differences of eluted NGF from composite materials and PEGPCL hydrogels indicate that composite materials significantly reduce NGF loss in the early release phase (“burst” release) and also extend the NGF release profile. Also, it is possible that the fabrication method for composite materials might better preserve NGF activity than that of PEGPCL hydrogels.

Since NGF release profiles from composite materials are closely related to the area of PEGPCL hydrogel covered by EFMs; longer release duration can easily be obtained by changing the size and thickness of EFMs or by applying patterning techniques (e.g., photolithography) to reduce the exposed hydrogel area. NGF was eluted from composite materials at a higher concentration than from PEGPCL hydrogel boluses at each time point, especially in the first week, demonstrating a remarkable reduction of NGF loss in the early release cycle (< 2 days). This indicates slower diffusion rates and degradation profiles, most likely resulting from the presence of EFMs.

6.2.4 Characterization of eluted NGF bioactivity through PC12 cell neurite extension

The quality of NGF eluted from hydrogel-EFM composite electrode coatings was evaluated using PC12 cell neurite extension. Composite electrode coatings delivered the targeted amount of bioactive NGF for over 2 weeks. Neurite extension of PC12 cells exposed to samples was characterized at day 7 and 14. At day 14, neurite extension was still evident (Figure 50A, NGF positive control and 50B, coating), indicating that sufficient, bioactive NGF was released from composite electrode coatings to elicit a neuronal phenotype. The neurite number and length were similar to that of a positive control (which received 50 ng/mL NGF every other day for 14 days). Also, PC12 cells exposed to a negative control (no NGF) and a sham (BSA-releasing coatings) displayed rounded, undifferentiated morphologies at day 14 (Figure 50C, and D), consistent with the expected morphology of PC12 cells not exposed to NGF.

Neurite length distributions at day 7 and day 14 of PEGPCL hydrogel-PCL EFM composite electrode coatings were statistically insignificant ($p > 0.05$) from those of NGF

positive control samples (Figure 51A, and B). These results were consistent with the observations in Figure 50.

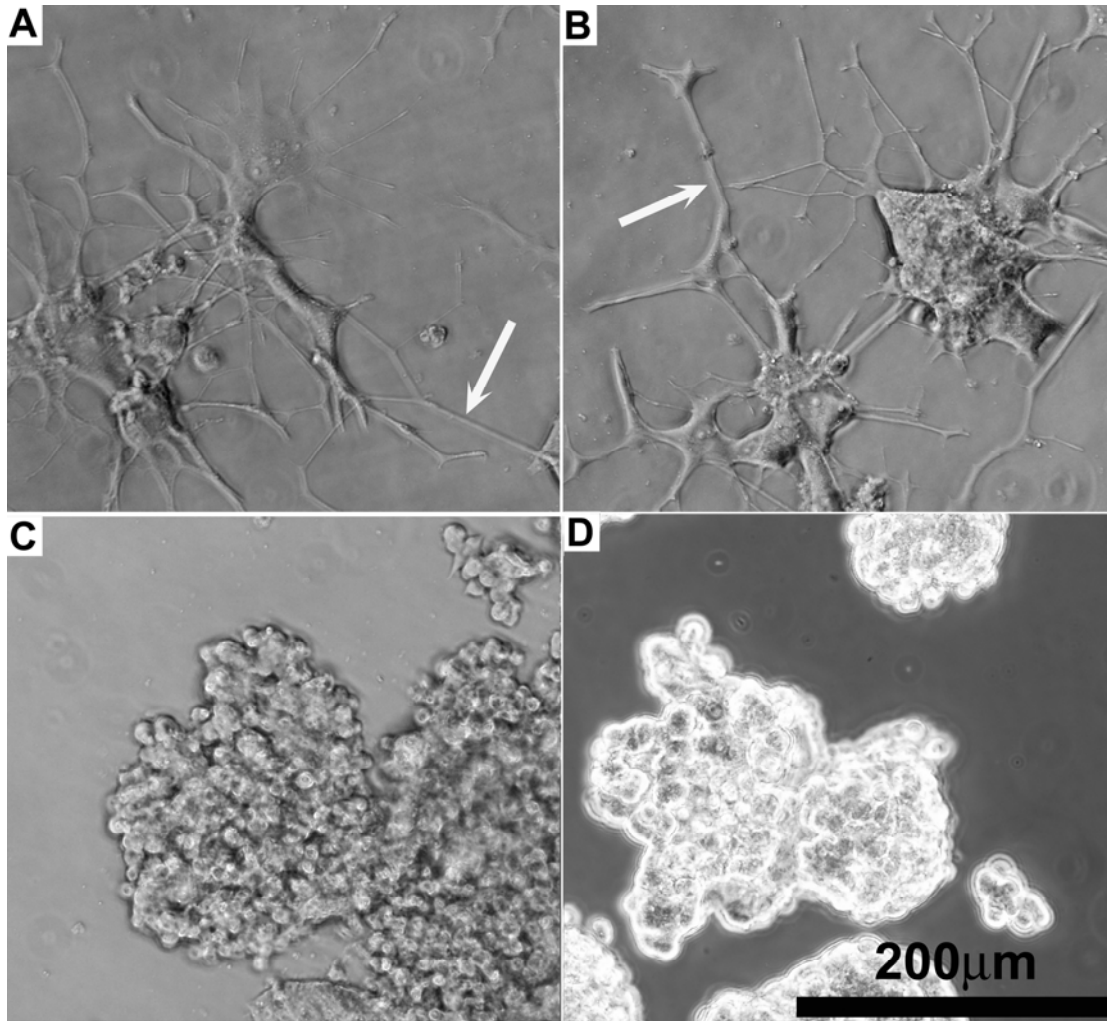


Figure 50. Representative phase contrast optical micrographs of PC12 cells after 14 days cell culture. (A) Positive control receiving 50 ng/mL NGF in the media. (B) NGF-eluting composite coating as shown in Figure 45. (C) Negative control receiving 0 ng/mL NGF. (D) Sham, BSA-releasing composite coating.

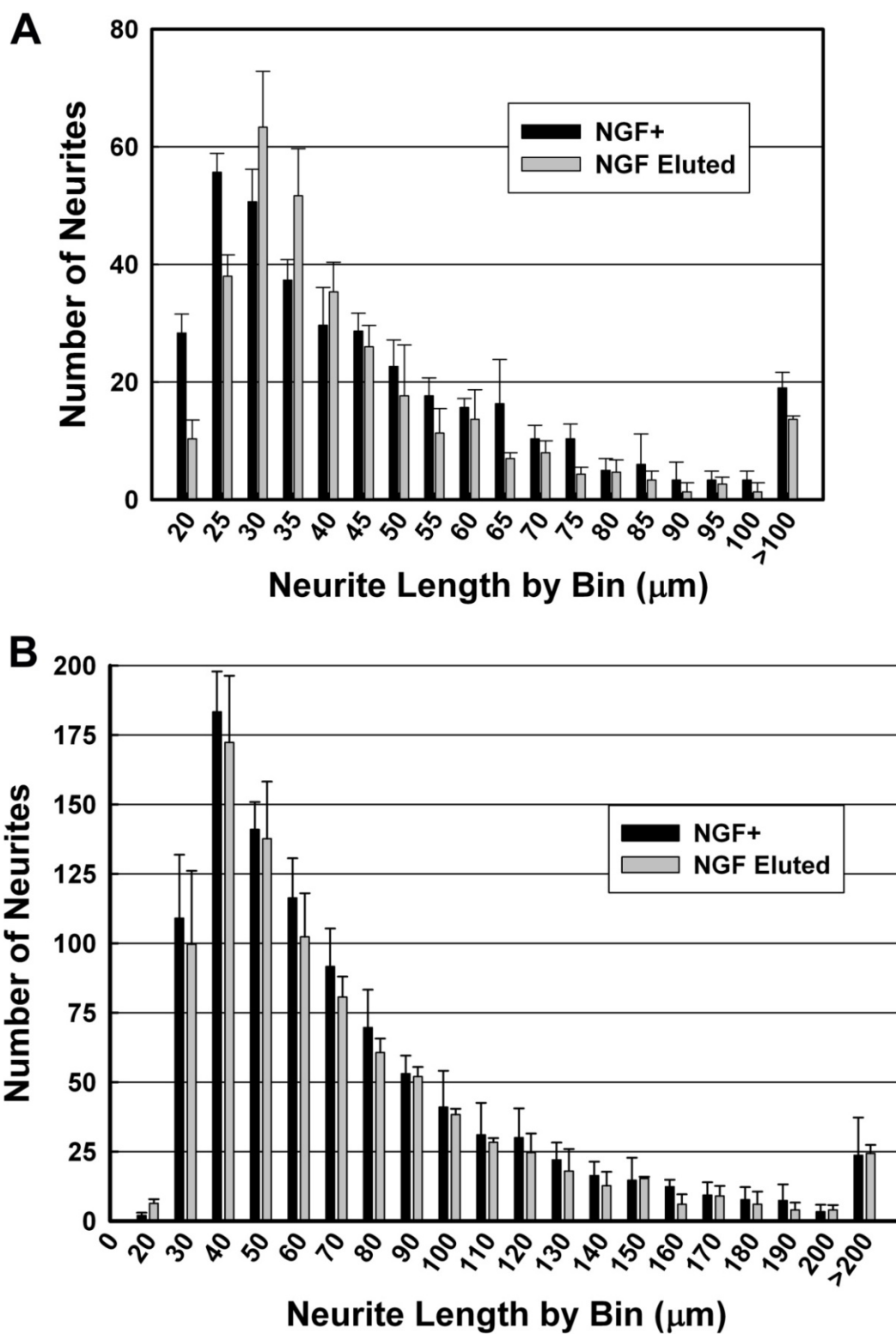


Figure 51. Histograms of neurite length after culture for (A) 7 and (B) 14 days. Black: NGF positive control; gray: NGF-eluting composite electrode coatings.

Also, for both composite electrode coatings and NGF+ control samples, neurite length distributions at day 14 were statistically different ($p < 0.05$) from those at day 7 (Figure 52), which reflected the sustained release of NGF from electrode coatings for over 14 days at a concentration sufficient to induce neurite extension. It should be noted that cells continued to extend neurites beyond the 14 day period investigated, but neurite length measurements beyond 14 days became difficult as a result of neurite branching and network formation in both NGF+ control and electrode coating samples. Qualitatively, neurite extension of composite electrode coating samples remained visibly similar to NGF+ control samples and distinct from that of negative control and sham samples beyond 14 days of cell culture.

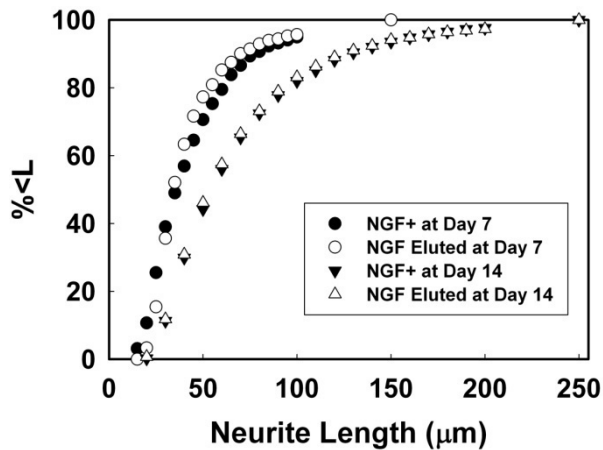


Figure 52. Percent of neurites possessing length $< L$ at day 7 and 14.

We have demonstrated that neurotrophin-eluting composite electrode coatings have the potential to enhance neuron proximity to devices. Coatings can induce neurite extension in PC12 cells for over 2 weeks (Figure 50B). Thus, composite electrode coatings could not only improve interaction between adjacent neurons and the device, but could also potentially impact cells several hundred microns from the electrode surface.

Apart from neurotrophin releasing systems, other methods have been proposed to enhance tissue-device contact, including the employment of passive surface coatings [56, 357, 358], which promote neurite extension and cell adhesion through direct contact to modified or patterned surfaces. However, with the potential to release soluble factors, PEGPCL hydrogel-PCL EFM composites offer the substantial advantage of influencing cells distant from the implant site. Additionally, compared with hydrogel coatings, the external EFMs in composite coatings can enhance cell adhesion as extensively explored in the past decade [315, 359], and demonstrated in Chapter 5. To further enhance adhesion, adhesion molecules (e.g. polylysine, collagen, laminin) could be used to modify EFM surfaces through standard bioconjugation methods. Thus, composite coatings, such as those presented here, that provide sustained, multiple (e.g., soluble and adhesive) cues hold tremendous promise in improving the neuronal tissue-electrode interface.

6.3 Conclusions

These results demonstrate that PEGPCL hydrogel-PCL EFM composite coatings could improve neural prosthesis biocompatibility by delivering neurotrophins at biologically significant concentrations in a controlled and sustained manner to stimulate neural survival and differentiation. The incorporation of PCL EFMs in hydrogel-based coatings can constrain the hydrogel bolus, providing an additional diffusion barrier and therefore extending both coating adhesion to electrode surfaces and neurotrophin release. These hydrogel-EFM composite coatings thus provide a promising approach to encourage neuronal sprouting towards device surface and enhance neural prosthesis performance over the long-term.

Chapter 7: Conclusions and Future Directions

7.1 Summary of dissertation

Although several strategies have demonstrated their efficiency in nerve regeneration of the PNS, the unique physiology of the CNS makes it challenging to achieve complete restoration of neural functions by the use of these strategies. As alternative approaches to help patients restore lost neural functions and recover from neurological diseases or traumas, neuroprosthetic devices have attracted significant attention in the past decade. However, when applied in the CNS, neuroprosthetic devices frequently fail to maintain stable and reliable performance long-term as a result of the complex acute and chronic immune reactions mounted against the implanted electronic components (e.g., electrodes and neural probes). Thus, neuron-electronic device interfaces have been extensively studied to increase the interface biocompatibility and reduce the formation of glial scar. Current progress includes the development of microelectrode arrays made of different materials (e.g., stainless steel, silicon, and polyimide), in various configurations (e.g., microwires, “Michigan arrays”, and “Utah arrays”), the study of the effect of implantation techniques on device performance, and the incorporation of haptotactic and chemotactic cues into the interface to improve neuron-electronic device interaction.

We present a novel composite material comprised of PEG-polyester hydrogels and PCL-based electrospun fiber mats, which combines the advantages of both hydrogels

and EFMs. As potential tissue engineering constructs and controlled drug delivery systems, our hydrogel-EFM composite materials can provide contact guidance and essential drugs simultaneously, and therefore can be applied as neurotrophin-releasing electrode coatings to reduce the acute and chronic inflammation and enhance electrode long-term performance.

To create hydrogel-EFM composite materials, we synthesized PEGPCL and PEGPLA diacrylate copolymers for hydrogel formation, and fabricated PCL EFMs (**Chapter 3**). ^1H NMR and FT-IR were applied for polymer characterization, and the calculated yields of acryl-PEGPCL and acryl-PEGPLA were 83.7% and 85.4% respectively. The hydrophobicity of PCL EFMs was modified through plasma treatment (fluorinated PCL and acrylic acid-treated PCL EFMs) or core-shell co-electrospinning (PCL/PEGPCL core/shell EFMs). Also, by varying the fiber deposition time, PCL EFMs with different thicknesses (300 μm , 800 μm , and 1100 μm) were obtained. For all types of EFMs, several parameters were characterized, including water contact angle, average fiber width, mean pore size, and porosity. The results showed no significant difference in average fiber width, mean pore size, and porosity among all EFM samples. Water contact angle tests confirmed our hypothesis that fluorinated PCL EFMs were super hydrophobic (average 157.0° water contact angle), whereas acrylic acid-treated PCL and PCL/PEGPCL core/shell EFMs were super hydrophilic (water contact angles close to 0°). For PCL EFMs, surface hydrophobicity increased slightly from 135.2 ° to 149.6 °, as the thicknesses of EFMs increased from 300 μm to 1100 μm .

In **Chapter 4**, we investigated the effect of different composite material configurations on protein (BSA) release kinetics. Bulk PEGPCL hydrogels and PCL

EFMs were combined through UV photo-polymerization. Two sandwich-like composite materials were constructed: Composite A, with a PCL EFM in the middle of two hydrogel layers, and Composite B, with a PEGPCL hydrogel layer in the middle of two PCL EFMs. The comparison of the profiles of BSA released from different composite materials indicated that when applied as external layers, PCL EFMs could act as additional diffusion barriers, significantly reducing the initial burst release (from 20% to 7%) and providing a more linear release profile with increased release duration (from 40 days to 70 days). Aggregation status of BSA released from Composite B materials was evaluated using native PAGE, demonstrating only a $\sim 9\%$ increase in BSA aggregation after 70 days release. Biocompatibility and bioactivity of Composite B materials were studied using a PC12 cell model. After 2 weeks cell culture, no significant difference of cell viability was found between our composite material and negative control samples. Also, our Composite B materials were capable of delivering active NGF for at least 14 days, stimulating neurite extension in PC12 cells.

It is likely that the incorporation of external PCL EFM layers not only increased the protein diffusion path length, but also altered the hydrogel swelling, hydrogel degradation rate, and the hydro-phobicity/philocity interaction between the dissolved drugs and the scaffold network. To test our hypotheses, we coupled PEGPLA hydrogels (which degrades faster than PEGPCL hydrogels) with EFMs of different thicknesses or hydrophobicity in the Composite B configuration. The investigation of the swelling behavior of our composite materials indicated that while the addition of external EFM layers appeared to constrain swelling in the radial direction (r) and increase axial swelling (z), composite materials comprised of thinner or more hydrophilic EFMs

displayed reduced swelling, probably because of the formation of a better interface between hydrophilic hydrogel and thinner or more hydrophilic EFMs. The study of BSA release kinetics confirmed our hypothesis that both the thickness and hydrophobicity of EFMs employed in composite materials contributes to protein release behavior. Smaller burst release and extended, more linear release profiles can be obtained by increasing either the thickness or hydrophobicity of external EFMs.

In **Chapter 5**, we studied the effect of topography and hydrophobicity of external EFMs layers on cell adhesion and morphology. SK-N-SH cells were cultured overnight on six different samples (PEG hydrogels, PEGPCL hydrogels, PEGPCL hydrogel-PCL EFM composite materials, PEGPCL hydrogel-PCL/PEGPCL core/shell EFM composite materials, PCL EFMs, and PCL/PEGPCL core/shell EFMs). The results of MTT cell adhesion tests and fluorescent imaging showed that while there was significant difference of SK-N-SH cell adhesion between each category (hydrogel < composite material < EFM), no significant difference was found in each category, indicating that it was the contact guidance (e.g., topographical and mechanical properties) provided by different scaffolds that determined the cell adhesion status. The morphology of the SK-N-SH cells cultured on composite materials and EFMs was characterized by SEM. Although cells on EFMs exhibited lower circularity than those on composite materials, no significant difference of cell spreading area was found between composite material and EFM samples. In general, the incorporation of external EFM layers can significantly increase the cell adhesion ability of PEG-based hydrogel systems. Also, the study of rat cortical cells demonstrated that cortical cells preferred to adhere to the composite materials with hydrophilic EFMs.

Next, we investigated the efficacy of hydrogel-EFM composite materials as coatings at the neuroprosthetic interface (**Chapter 6**). Coating adhesion ability was studied through the PBS immersion test, agarose gel phantom test, and insertion and removal test. The results showed that composite coatings could remain at the electrode surface after insertion and removal into a tissue phantom, and coating adhesion was observed for over 9 months in the PBS immersion test, and 1 month in the agarose gel phantom test. The evident degradation or delamination of the hydrogel layer, which was found in the hydrogel region not covered by the EFM layer, indicated that the presence of the external EFM layer constrained the hydrogel layer underneath, reducing the degradation of the hydrogel, and significantly increasing coating potential to withstand compression or shear forces. Also, no significant cytotoxicity of composite coatings was found in a PC12 cell culture test. Composite coatings displayed NGF release for ~ 25 days at concentrations higher than or close to the NGF effective dose (50 ng/mL), and eluted NGF bioactivity was characterized through PC12 cell neurite extension experiments. Similar PC12 cells morphology and neurite extension was observed in both NGF-eluting composite coating samples and NGF-positive controls, confirming the effectiveness of composite coatings.

7.2 Conclusions

These results demonstrate that PEG-polyester hydrogel-EFM composite materials can be formed through UV photo-polymerization and applied as coatings for neuroprosthetic devices. Besides improved electrode coating adhesion, composite coatings can provide controllable neurotrophin release that may ultimately minimize the acute immune response and facilitate the formation of a stable interface between the

implanted electronic component and surrounding tissue. Moreover, these composite materials have far-reaching applications.

The combination of these two biomaterial matrices represents a new strategy in drug delivery, yielding a remarkably versatile system. Both systems have been previously employed independently as drug delivery vehicles and their properties can be altered by adjusting defined parameters (e.g., polymer selection, crosslinking density, voltage, electrospinning time). We have shown here that the combination of hydrogels and EFMs produces a novel release system with controllable, emergent properties. For example, the composite diffusion coefficient can be adjusted by decreasing the EFM pore size, which is dependent on network porosity (ϵ), fiber density, and fiber diameter (width, ω) [334]. Thus, smaller pore sizes, and lower drug diffusion rates, should be obtainable by increasing fiber deposition time or reducing polymer solution flow rate. EFMs with a wide range of fiber fractions, pore sizes, and pore size distributions are already available [46, 47]. Also, because of the close relationship between polymer material and fiber density, release profiles can be adjusted by changing the electrospun polymer composition [360]. Thus, the composite diffusion coefficient could be easily adjusted by altering electrospinning synthesis parameters without the need for extensive chemical modification. Also, the resulting therapeutics release profiles can be adjusted by the composite configuration and EFM thickness and hydrophobicity as demonstrated in Chapter 4.

Additionally, when EFMs are used as external layers in composite materials, new topographical cues can be introduced into the materials, improving cell-scaffold interactions (e.g., cell adhesion, spreading, migration, and proliferation) by mimicking

the structure of ECM [282, 316, 361]. Thus, these composite materials have great potential in tissue engineering applications. Also, the techniques developed for the improvement of hydrogel biological properties or EFM fabrication and surface modification, can all be applied to composite materials, further broadening their biomedical applications.

7.3 Future directions

7.3.1 Simultaneous and directional release of different drugs

The design of hydrogel-EFM composite materials is extremely versatile and should easily permit anisotropic compound release. For example, anisotropic release could be achieved by using aligned fibers to apply mechanical strain [362], producing an anisotropic diffusional barrier. Alternatively, anisotropic release could be accomplished by changing the number and/or position of EFMs in the composite system. Thus, multiple hydrophilic active biomolecules could be separately introduced into different hydrogel layers (divided by EFMs) to yield different release profiles achieved simultaneously or with directional selectivity. As demonstrated in Figure 53, BSA and NGF could be incorporated in separate PEGPCL hydrogels, which fill two chambers of a cylindrical composite material system, vertically separated by a piece of EFM in the center. This design restricts the direct release of NGF and BSA to only a half volume of the surrounding HA gel (which models tissue), resulting in directional protein release. Since NGF is effective at stimulating PC12 cells neurite extension, and this phenomenon is NGF dose-dependent; PC12 cells seeded at different positions inside the HA gel should exhibit different morphologies over time. Cells which are closer to the NGF-containing chamber should display longer neurites than those closer to the BSA-containing chamber.

Also, the initially uniform PC12 cell distribution inside HA gel may be altered, because of potential cell migration towards the higher NGF concentration region and also settling induced by gravity.

The significant differences in both protein diffusion coefficients and hydrogel degradation rates in aqueous solutions versus tissue-like gels should be taken into account when constructing this model. Copolymers with higher molecular weight and more degradable ester units, or hydrogels with lower crosslinking density, may be required to increase drug release rate and hydrogel degradation rate inside HA gels.

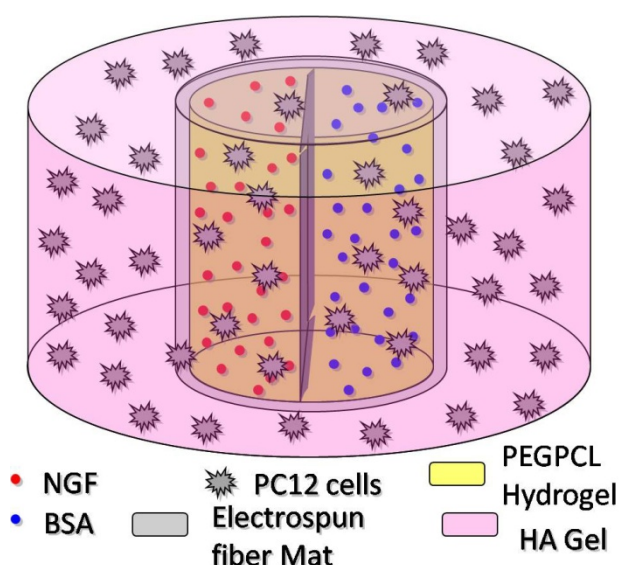


Figure 53. Schematic of simultaneous and directional release of BSA and NGF in PC12 cell culture model

7.3.2 Dual release of hydrophilic and hydrophobic drugs

We demonstrated that composite materials are promising candidates for hydrophilic drug controlled release in Chapter 4. However, in some cases, sustained release of hydrophobic drugs (e.g., cancer drugs and anti-inflammatory therapeutics) is desired in clinical applications. This technology has the potential to mediate dual release

of hydrophilic and hydrophobic drugs. Since drug solubility in the delivery system plays an important role in release rate [281, 363]; hydrophobic proteins/drugs, which are more compatible with hydrophobic polymers (e.g., PCL), could be introduced into the EFM phase, whereas a hydrophilic component could be introduced into the hydrogel phase. The distribution and loading efficiency of hydrophobic macromolecules can be greatly improved in this way. For example, we could introduce dexamethasone into PCL fibers through electrospinning, and construct NGF-containing PEGPCL hydrogel-dexamethasone-containing PCL EFM composite coatings for neuroprosthetics. The dual release of NGF and dexamethasone from the composite coating could attract nearby neurons to extend neurites towards the electrode site and reduce inflammation simultaneously, further promoting the integration of tissue and the implanted electrode. Thus, the applicability of hydrogel-EFM composite electrode coatings can be greatly enhanced through the dual release of hydrophobic and hydrophilic drugs.

7.3.3 Surface modification of composite materials

Further, hydrogel-EFM composite materials have several additional advantages for biological application. Since cell behavior is critically affected by environmental factors, such as topographical patterns and surface chemistry, a number of strategies can be applied to enhance cell-scaffold interaction through modifications of the EFMs. First, EFMs can be easily modified either by direct surface modification [253] or core-shell synthesis with biomolecules to improve tissue integration [241, 364]. Second, EFMs can be easily altered via micropatterning through UV photolithography [356] or laser ablation [355] to provide new micro-scale topographical features (e.g., linear grooves and microwells) for cell attachment and proliferation. In addition, this method could be used

to pattern composite electrode coatings to reveal electrode sites in MEAs. Thus, MEA conductivity and electrical signal transmission would not be compromised by the addition of a patterned composite coating.

7.3.4 A look to the future

As described in Chapter 1, the primary cause of electrode failure in a chronic setting is the formation of an encapsulating sheath around the implanted electrode, comprised of compacted astrocytes, microglia, oligodendrocytes, cell debris, and blood vessels. Studies of the interaction between neurons and composite coatings are just the start of tissue-device interface investigation. Complementary experiments of the response of glial cells, especially astrocytes, to composite coatings should be conducted to test the efficacy of reducing glial scar formation in the first 1-3 weeks post-implantation. To fulfill this goal, anti-inflammatory therapeutics could be introduced into electrospun fibers, while specific EFM surface modifications, such as aligned fibers [274] could be used to selectively attract neurons and repel astrocyte adhesion. Last, to have a better understanding of the interaction between composite coatings and the surrounding tissue, *in vivo* studies should be performed following all of these *in vitro* investigations.

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